

**THE ROLE OF OVINE BETARETROVIRUSES
IN UTEROPLACENTAL FUNCTION**

A Dissertation

by

KATHRIN ANSON DUNLAP

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Physiology of Reproduction

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ABSTRACT

The Role of Ovine Betaretroviruses in Uteroplacental Function. (August 2006)

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Co-Chairs of Advisory Committee: Dr. Thomas E. Spencer
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Endogenous retroviruses (ERVs) account for a substantial portion of the genetic pool of every animal species (e.g. $\sim 8\%$ of the human genome). Despite their overwhelming abundance in nature, many questions on the basic biology of ERVs are unanswered. Sheep harbor approximately 20 copies of endogenous betaretroviruses (enJSRVs), which are related to an exogenous oncogenic virus, Jaagsiekte sheep retrovirus (JSRV). Therefore, they are an attractive model for investigation of the potential beneficial roles of ERVs in reproductive biology.

Studies were conducted to determine: 1) expression of enJSRVs envelope (*env*) and *HYAL2* mRNAs in the ovine uterus and conceptus (embryo/fetus and extraembryonic membranes) throughout gestation; 2) regulation of enJSRVs expression by progesterone; and 3) the role of enJSRVs in regulating peri-implantation placental growth and differentiation.

Study One determined the localization of enJSRVs *env* and *HYAL2* mRNAs throughout gestation. Results demonstrate that alterations in expression of enJSRVs and *HYAL2* in the sheep uterus and placenta suggest the probability of a variety of physiological roles in implantation and placentation. Partial sequencing of the

transcriptionally active enJSRVs from ovine uteroplacental tissues revealed expression of multiple enJSRV loci.

Study Two assessed the influence of progesterone, interferon tau, and pregnancy stage on enJSRVs expression, as an effort to understand factors that may regulate enJSRVs. Results of this study support the hypothesis that expression of enJSRVs is modulated by progesterone, but not IFN τ in vivo.

Study Three provides for enJSRVs regulating trophoctoderm growth and differentiation in the peri-implantation conceptus. Blocking conceptus enJSRVs Env expression compromised pregnancy by retarding trophoblast outgrowth and differentiation. Inhibition of enJSRVs Env in vitro also reduced proliferation of mononuclear trophoctoderm cells. Consequently, these results demonstrate that enJSRVs Env regulates trophoctoderm growth and differentiation in the ovine conceptus, strongly supporting the biological significance of ERVs in placental evolution and animal reproduction

Collectively, these studies illustrate that enJSRVs play an integral role in success of pregnancy. While the definitive roles of the enJSRVs have not yet been elucidated, it is evident that enJSRVs are an important component of the ovine genome and that they influence recognition and maintenance of pregnancy and placental formation.

DEDICATION

To my family

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I would like to thank my committee chairs, Drs. Thomas E. Spencer and Fuller W. Bazer for their guidance and support throughout the course of this program as well as their example of excellence. I am also most appreciative of the encouragement provided by my committee members, Drs. Robert C. Burghardt, Greg A. Johnson, and David J. Caldwell.

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Thank you does not seem a strong enough statement to offer to my family. They are, now and forever will be, my driving force and greatest treasure. It is their love and understanding that makes anything possible and everything worthwhile.

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CHAPTER I

INTRODUCTION

Maternal recognition and establishment of pregnancy requires that the trophoblast layer of the blastocyst produce pregnancy recognition hormones, such as chorionic gonadotropin (CG) in the human and interferon tau (IFN τ) in domestic ruminants, that ensure continued production of progesterone, the hormone of pregnancy, by the corpus luteum [1]. In humans, rodents and ruminants, the mononuclear cytotrophoblast cells must differentiate into a syncytiotrophoblast or analogous structure that creates a maternal-fetal interface necessary for the conceptus to derive hematotrophic nutrition from the mother [2]. Defects in production of pregnancy recognition signals and in trophoblast differentiation are associated with pregnancy complications and loss in humans as well as domestic ruminants [3-5].

Successful placental morphogenesis is also critical for fetal survival. The placenta is a complex organ that provides for transport of nutrients for the fetus, manages waste products, regulates gas exchange, and suppresses immunological rejection by the mother. In sheep, placentation requires the differentiation of mononuclear trophoblast (trophoblast) cells into binucleate cells (BNC), which are speculated to then invade and fuse with the LE to form multinucleated syncytiotrophoblasts. Although critically important for fetal survival, the cellular and molecular mechanisms governing placental morphogenesis are generally not known and are severely understudied.

This dissertation follows the style of Biology of Reproduction.

An attractive hypothesis is that trophoblast growth and differentiation into BNC and formation of multinucleated syncytiotrophoblast involves endogenous retroviruses (ERVs), specifically endogenous Jaagsiekte sheep retroviruses (enJSRVs) and HYAL2, the cellular receptor for JSRV and enJSRV Env [6, 7]. Endogenous retroviruses are integrated into the germline of most, if not all, eukaryotes. While their presence is accepted, their biological role is not well understood. Preliminary evidence suggests that endogenous JSRVs in the ovine uterus regulate conceptus-endometrial interactions, conceptus development and implantation, as well as placental morphogenesis. Continued investigation into these phenomena is required in order to generate fundamental knowledge concerning the cellular and molecular mechanisms of trophoblast growth and differentiation, as well as the basic mechanisms of cell-cell fusion.

CHAPTER II

LITERATURE REVIEW

Retroviruses

Retroviruses are a diverse family of RNA viruses that are widespread in nature and infect most vertebrate and many non-vertebrate species. The initial discoveries of retroviruses predominantly resulted from studies on tumor-causing viruses [8-11]. However, retroviruses are capable of inducing many different diseases beside cancer, such as immunodeficiencies, neuropathies, pneumonia, and diseases of bone and joints. Despite the diversity of species infected, routes of transmission, and disease manifestations, all retroviruses are quite similar in terms of virion structure, genomic organization, and replication cycle.

All retroviral genomes contain four main genes encoding virion proteins usually in the same order: 5'-gag-pro-pol-env-3' [12]. The gag gene encodes the matrix (MA) protein, the major capsid (CA) protein and the nucleocapsid (NC) protein. The pro gene encodes a protease (PR) while the pol gene encodes the enzymes reverse transcriptase (RT) and integrase (IN). The envelope gene encodes the envelope glycoproteins, both SU (surface) and transmembrane (TM) [12].

Upon entry into an infected cell, the RNA genome of retroviruses is reverse transcribed into double stranded DNA [13, 14] and subsequently integrated into the genome of the host cell (termed provirus at this stage) [15-18]. Stable integration of retroviruses into the genomic DNA of the host cell has allowed them to colonize the germ line of virtually all vertebrates. Once integrated into the germ line, ERVs are

inherited by the host vertically in a Mendelian fashion, and are termed “endogenous” retroviruses. In contrast, “exogenous” retroviruses (e.g. human immunodeficiency virus or HIV) are transmitted horizontally from infected to uninfected host similarly to other viruses [19].

Endogenous Retroviruses (ERVs)

A unique feature of retroviruses is their presence as inherited elements in the germline of most eukaryotes. These elements, known as endogenous retroviruses (ERVs), are transmitted through the germline as stable Mendelian genes, yet they exhibit structural and sequence similarities to infectious exogenous retroviruses [20]. Generally, endogenous proviruses are transcriptionally silent and are often defective, typically differing from the exogenous counterpart by deletions or point mutations that render them incapable to form infectious virus. However, several ERVs maintain at least some intact open reading frames with expression associated with either beneficial or detrimental effects to the host [20]. The biological relevance of ERVs in mammals has been intensely debated. ERVs have been characterized either as dispensable junk DNA or as indispensable for mammalian development and physiological function [21-23].

ERVs are classified as “ancient” or “modern” depending on the length of time from presumed entry into the genome. Ancient viruses were acquired in evolutionarily “ancient” times, as indicated by their identical integration in more than one species. Humans were believed to contain only ancient ERV, but a full length and potentially replication competent ERV (HERV-K113) was isolated from some human populations, suggesting that this element is not yet fixed in the genetic pool and might still be able to

re-infect humans [24]. ERVs appear to be initially derived from exogenous retroviruses that integrated into the germ line of a particular animal and slowly became fixed in the gene pool of that species. After this step, ERVs and their exogenous counterparts evolved along two different “tracks”. ERVs, stably residing in the germ line, were transmitted as Mendelian genes, whereas the exogenous viruses were maintained in the environment only by successful infection of new hosts. Thus, ERVs were subjected to far less evolutionary pressure than the exogenous viruses. Ancient ERVs do not appear to have exogenous counterparts.

In recent years, considerable effort has been directed toward understanding the biological significance of ERVs, particularly those present in the human germline [21-23]. Specific expression of some ERVs in the placenta has lead to various hypotheses that these elements play a role in mammalian reproduction [25-33].

ERVs in the Placenta

In the 1970s and 1980s, expression of ERVs was detected in the placentae of various animal species, including primates, cats, mice, guinea pigs, Syrian hamsters [28, 34-36], as well as humans [37, 38]. Expression of ERVs in the genital tract and placenta of various animal species has been described for at least three decades [28, 34-38], sparking opinions on their significance in the evolution of placental mammals and development of viviparity [27, 33, 39, 40]. The evolutionary significance of these observations has been debated for years. Indeed, the hypothesis that ERVs have a fundamental biological role in placental morphogenesis was proposed over a decade ago [28].

Several HERV families, HERV-F, HERV-FRD, HERV-K (HML-2), HERV-R

(ERV3), HERV-T, and HERV-W are active in human placenta and code for intact retroviral Env proteins [41], but few of them have been studied in any detail. ERV3 is detectable in many primate species suggesting that they are conserved throughout primate evolution[32]. Recent research has demonstrated that ERV3 is also highly expressed in the syncytiotrophoblast [32]. The similarity of the transmembrane (TM) region of ERV3 with a putative immunosuppressive region, termed p15E of gammaretroviruses [42], lead to speculation that ERV3 may protect the fetus from immune attack by the mother [32]. As reviewed recently by Rote and colleagues [43], the amino acid sequence of the ERV3 Env is highly unusual when compared to similar proteins from infectious retroviruses. Specifically it lacks a leader sequence, a membrane-spanning domain, and a fusion peptide. Additionally it is largely insensitive to proteolytic generation of separate surface (SU) and TM subunits. Available results suggest that ERV3 Env initiates production of the β -subunit of CG and commits the trophoblast to cell cycle arrest [44]. However, a great deal of additional investigation must be performed before ERV3 can be either implicated or dismissed as an initiator of trophoblast differentiation [43].

The HERV-W Env does not contain a p15E-like immunosuppressive region, but does contain a fusion peptide region near the furin cleavage site between the SU and TM domains. The product of the HERV-W envelope (*env*) gene is a fusogenic membrane glycoprotein termed syncytin-1, which elicits cell fusion in a variety of cell lines, including BeWo, COS and 293 cells [26, 29]. As in the fusion process of retroviral Env protein, syncytin binds its cognate receptor to initiate the fusion process. Currently, two

human sodium-dependent amino acid transporters, ASCT1 and 2, are purported to be the syncytin receptors [45]. However, a direct interaction between syncytin and these transporters has not been demonstrated. Indeed, a specific requirement of the ASCT receptor for syncytin-mediated cell fusion is not known. The Env protein of HERV-FRD, syncytin-2, also has fusogenic properties in a variety of mammalian cell lines [46]. Although syncytin-1 and -2 are fusogenic in a number of cell types, the products of other HERV *env* genes, such as *envV*, are not fusogenic in any cells tested, despite being expressed only in the placenta [47]. As reviewed by Rote and colleagues [43], intercellular membrane fusion is thought to be a multi-step process, requiring intercellular adhesion and drawing of membranes together by a fusion protein, concurrent with phosphatidylserine efflux. The result is membrane rearrangement and joining. In the human cytotrophoblast, HERV-W Env may be the principal fusion protein. The differential biological activities of the Env products of HERVs suggest that each ERV expressed in the trophoblast has a different function in placental morphogenesis.

Recently, Dupressoir and coworkers [48] used a systematic *in silico* search to identify syncytin-A and syncytin-B, which are two fusogenic placenta-specific murine *env* genes of retroviral origin conserved in Muridae. A phylogenetic analysis places them within the enlarged HERV-F/H family that also contains the human syncytin-1 and -2 *env* genes, but in branches distinct from the latter. The syncytin-A and -B mRNAs are expressed in the syncytiotrophoblast-containing labyrinthine placenta. Further, the mouse genome has many other genes (~15-20) that share high similarity to syncytins-A

and -B. The syncytin-A and -B genes trigger cell-cell fusion in *ex vivo* transfection assays, with distinct cell type specificities, suggesting different receptor usage. The receptor(s) for mouse syncytin-A and -B is not known. Syncytin-A and -B may play a critical role in syncytiotrophoblast formation. Thus, two pairs of ERVs, independently acquired by the primate and rodent lineages, may have been positively selected for a convergent physiological role in syncytiotrophoblast formation. Indeed, placental syncytial cells have originated on multiple independent occasions during mammalian evolution, being observed in Artiodactyla, but not in pigs or camels, and in Carnivora, Rodentia, and Primata [49]. Collectively, available results from studies in humans, mice and sheep support the hypothesis that several independent retroviral infections may have contributed to the emergence of a common syncytial barrier in different species and played a pivotal convergent role in placental morphogenesis and physiology [6, 27, 39, 48].

Jaagsiekte Sheep Retrovirus (JSRV)

Jaagsiekte sheep retrovirus (JSRV) is an exogenous and pathogenic retrovirus of sheep. JSRV is the cause of ovine pulmonary adenocarcinoma (OPA, also known as jaagsiekte or sheep pulmonary adenomatosis), a major infectious disease of sheep [50-53]. Curiously, “Dolly”, the ewe who was the first mammalian cloned by nuclear transfer, died as a result of JSRV infection (Ian Wilmut, personal communication). The JSRV genome has a simple genetic organization, characteristic of the replication-competent betaretroviruses [54]. The Env glycoprotein complex of retroviruses includes two polypeptides, an external, glycosylated hydrophilic polypeptide (SU) and a

membrane-spanning protein (TM), that together form an oligomeric knob or knobbed spike on the surface of the virion.

JSRV is the only virus that induces lung cancer. JSRV induces transformation of the differentiated epithelial cells of the lungs (type II pneumocytes and Clara cells). The JSRV long terminal repeat (LTR) (where the viral promoter and enhancers are located) is preferentially active in differentiated epithelial cells of the lungs and interacts with lung-specific transcription factors [55, 56]. Thus, the restricted expression of JSRV in type II pneumocytes and Clara cells is due to the preferential activation of the JSRV LTR in these cells. Of particular note is the fact that expression of the JSRV Env is sufficient to induce cell transformation *in vitro* by mechanisms involving activation of the PI-3K/Akt and the Ron tyrosine kinase pathways [57-59].

Another exogenous betaretrovirus related to JSRV is ENTV (enzootic nasal tumor virus) [60, 61]. The genomic structure of ENTV is very similar to the highly related JSRV, including *in vitro* transformation capacity and receptor useage. Specifically, JSRV and the ENTV Env can transform rodent fibroblasts *in vitro* and both use of hyaluronidase-2 (Hyal2) as the cellular receptor [62, 63].

Hyaluronidase 2 (Hyal2) Receptor

The JSRV envelope receptor is hyaluronidase 2 (Hyal2), a glycosylphosphatidylinositol (GPI)-anchored protein [63, 64]. HYAL2 is a member of a large family of hyaluronoglucosaminidases, but exhibits very low hyaluronidase activity [64-66]. Research has demonstrated that Hyal2 serves as the point of entry for the JSRV/ENTV particles and also acts as a receptor for the ovine endogenous

betaretroviruses (enJSRVs) envelope [63, 64, 67]. Recently, we found that the HYAL2 mRNA was expressed solely in the BNC and multinucleated syncytia of the sheep placenta, but not in the uterus (endometrial epithelia, stroma, or myometrium) [7]. The specific role of Hyal2 at the maternal-fetal interface and the resulting effect on enJSRV action has not yet been elucidated. This is an important question with respect to the involvement of enJSRVs in ovine reproduction.

Ovine Endogenous Betaretroviruses (enJSRVs)

The sheep is a unique model to evaluate the possible impact of ERVs and their interaction with exogenous retroviruses during placental development. The ovine genome contains 15 to 20 copies of endogenous beta retroviruses (enJSRVs) that are highly related to the oncogenic exogenous beta retrovirus, JSRV and ENTV as determined by Southern blotting hybridization [68, 69]. No differences in the number or restriction profiles of enJSRVs were noted in various breeds of domestic sheep (*Ovis aries*) or in wild sheep (*Ovis spp.*) [68]. Therefore, *enJSRV* loci were fixed in the genome early in the evolutionary history of domestic sheep prior to the divergence of these species.

Of the multiple enJSRVs present in the genome only three have been sequenced: enJS56A1, enJ5F16, and enJS59A1 [70]. Each of these enJSRV loci share the genomic structure of JSRV, but each possesses a mutation and/or deletion that renders them individually incapable of forming infectious viral particles. Interestingly two other enJSRV loci, enJS5F16 and enJS56A1, do possess an intact Env, but neither are capable of forming virus particles or independently transforming cells like the exogenous JSRV

Env [70, 71]. Additionally, the enJS56A1 Env can bind to the HYAL2 receptor similar to the JSRV Env but the enJS56A1 Env does not activate the PI3K/Akt pathway, due to an in-frame deletion of a Y-X-X-M motif present in the JSRV Env, which is critical for PI3K/Akt (phosphatidyl inositol 3-kinase) activation [67, 71].

Overall the intracellular pathways and biological effects of enJSRV Env are not well known. A motif scan of the inferred amino acid structure of two known enJSRV Env (enJS56A1 and enJS5F16) revealed a high degree of similarity to the gp36 Env protein from a large number of retroviruses such as mouse mammary tumor virus (MMTV) and HERVs. Analysis of the enJS56A1 and enJS5F16 Env sequences together with their hydrophobic profiles indicates the presence of characteristic features of retroviral envelopes [72], including a canonical cleavage site (consensus: R/K-X-R/K-R) [73] between the SU and TM moieties of the proteins and the presence of hydrophobic domains corresponding to the fusion peptide and the TM domain. Both polypeptides are encoded by the *env* gene and are synthesized in the form of a polyprotein precursor that is proteolytically cleaved by furin during its transport to the surface of the cell. These proteins are not required for the assembly of enveloped viral particles, but they do have an essential role in the entry process. The SU domain binds to a specific receptor molecule on the target cell, which is HYAL2 for both JSRV and enJSRVs Env [64, 67]. The binding of the Env and receptor appears to activate the fusion of the TM protein and, by a process that remains largely undefined, subsequent cell-cell membrane fusion.

enJSRVs in the Female Reproductive Tract

While the exogenous pathogenic viruses, JSRV and ENTV, appear to have a strict tropism for secretory cells of the respiratory tract [70, 74, 75], enJSRV expression is most evident in the reproductive tract, specifically being localized within the epithelia of oviduct, uterus, cervix and vagina [70, 76, 77]. Endogenous JSRV RNAs are highly expressed in the uterine epithelia [76, 77]. It has been demonstrated that enJSRV RNAs are the most abundant RNA population in the entire endometrium [77]. The localization and abundance of enJSRVs within the uterus suggests a beneficial role to the host in terms of reproduction. Expression of enJSRV RNAs in the ovine uterus was initially identified by differential display PCR and PCR-based subtraction hybridization experiments [77]. In situ hybridization analyses revealed that enJSRV RNA expression was restricted to the endometrial luminal (LE) and glandular epithelia (GE). Indeed, the relative expression levels of the enJSRVs in the uterine endometrial epithelia is very high in comparison to a number of other genes expressed in the same epithelia as well as expression of enJSRVs in other sheep tissues [76-78]. Possible physiological functions include pregnancy recognition signaling by trophectoderm, and regulation of conceptus development and placental morphogenesis [76, 77, 79].

Ovine Blastocyst Development and Implantation

In all species, implantation of the blastocyst precedes and is required for successful placentation. It is a highly coordinated process that involves blastocyst hatching and shedding of the zona pellucida, pre-contact blastocyst orientation, apposition and attachment, and adhesion of the trophoblast to the endometrial luminal epithelium (LE)

[80, 81]. In sheep, the fertilized oocyte develops into a morula in the oviduct and then is transported from the oviduct into the uterus on Day 4 (post-fertilization) surrounded by the acellular zona pellucida [4, 81]. The morula develops into a blastocyst (205 μm) by Day 6, which hatches from the zona pellucida between Days 7 and 8, and then develops into a spherical or tubular form (340 μm by 425 μm) by Day 11 [4]. Between Days 12 and 16, the trophoblast of the blastocyst rapidly elongates to a filamentous form of 190 mm in length [4]. The mononuclear trophoblast cells of the elongating blastocyst produce $\text{IFN}\tau$, the ruminant pregnancy recognition signal [82]. The blastocyst is now termed a conceptus (embryo and associated extraembryonic membranes) [80]. As it elongates, implantation begins as the trophoblast apposes and transiently attaches to the uterine LE between Days 13 and 15. By Day 16, the conceptus begins to firmly attach and adhere to the endometrial LE [80].

In concert with trophoblast outgrowth during conceptus elongation, the trophoblast giant binucleate cells (BNC) begin to differentiate from the mononuclear trophoblast cells (MTC) in ruminants [83-87]. In the sheep conceptus, BNC first appear between Day 14 and 16, and remain detectable throughout most of pregnancy. By Days 16 to 18, they form 15-20% of the trophoblast where it is apposed to the uterine LE. Between Days 16 and 24, the uterine LE transforms to syncytial plaques. The BNC are considered analogous to extravillous cytotrophoblasts in humans and trophoblast giant cells in rodents with respect to polyploidy, migratory/invasive properties, lack of proliferation, and production of endocrine hormones [86, 88]. The mechanism by which BNC differentiate from mononuclear trophoblast cells is poorly understood. The

differentiation is accompanied by a loss of cytokeratin filaments and associated desmosomes [89-92] and altered expression of integrin subtypes [93, 94]. Available results suggest that cell adhesion may also be involved in the genesis and function of BNC [91, 94]. Evidence from studies of ultrastructure and serial semi-thin sections suggests that the mononuclear trophoblast cells exit the mitotic cycle followed by endoreduplication to generate BNC [95-97]. Alternatively, the BNC may form by fusion of mononuclear trophoblast cells. After formation, the BNC migrate to the microvillar junction and fuse with individual uterine endometrial LE cells, producing trinucleate fetomaternal hybrid cells [95]. Continued BNC migration and fusion with the trinucleate cells, together with displacement and/or death of the remaining uterine epithelial cells, is thought to produce the plaques of multinucleated syncytiotrophoblast that contain as many as 25 nuclei and cover each caruncle by Days 20-24 [96]. The caruncles are areas of the ovine endometrium that are aglandular and consist of only stroma covered by a single layer of LE. As reviewed by Cross and colleagues [88], the 'transport and barrier trophoblast' of the placenta of most mammals is organized into highly branched villous, tree-like folds termed cotyledons. The organization of the villi is one basis for classifying placental structures [98]. Rodents have a single cotyledon (discoid placenta), whereas humans have multiple cotyledons, though they are clustered into a single disc. In ruminants (sheep, cow), multiple cotyledons are scattered across the entire placental surface.

During pregnancy, the syncytium, in which no nuclear division has been found, expands enormously in area during formation of the placental cotyledons, presumably

accumulating increased numbers of nuclei from continued BNC differentiation, migration and fusion with the syncytia. The mechanism by which multinucleated syncytiotrophoblasts are formed from BNC fusion is poorly understood. The BNC and multinucleated syncytiotrophoblasts are an essential component of placental morphogenesis. Between Days 20 and 60, the cotyledonary areas of the placenta, which are formed predominantly by chorionic villi lined by syncytial plaques, begin to interdigitate with the maternal endometrial caruncles to form placentomes. The placentomes provide a source of hematotrophic nutrition for the fetus, because the maternal and fetal blood vessels are in very close proximity for exchanging oxygen and micronutrients. Failure of placentome development results in loss of the fetus during gestation [99]. The placentae of many other mammals contain syncytiotrophoblasts, which form the interface with the maternal blood stream by fusion of mononuclear cytotrophoblasts to form a multi-nucleated barrier [2]. The multinucleated syncytia of the sheep placenta are analogous in some respects to the multinucleated non-dividing cells of the syncytiotrophoblast in humans and rodents [49, 86]. In the human placenta, cytotrophoblasts exit the cell cycle and fuse together to form a multinucleated syncytium. The syncytiotrophoblasts of the human placenta are responsible for transporting nutrients and gases and produce endocrine hormones, such as placental lactogen [100]. Indeed, the BNC and multinucleated syncytia of the ovine placenta produce both peptide and steroid hormones, such as placental lactogen and progesterone, which are also produced by the human placenta [101].

Regulation of enJSRVs Expression by Progesterone

Investigation into the key regulatory mechanisms of enJSRV expression is necessary for understanding a potential relationship between IFN τ and enJSRVs. The use of transient transfection assays by Palmarini et al., [76] demonstrated that progesterone, acting via PR, increases transcription of enJSRV genes in vitro and transcriptional activity of several enJSRV LTRs. In situ hybridization of uterine cross sections further demonstrated that steady-state levels of enJSRV RNAs in LE and GE correspond to progesterone receptor (PR) levels, increasing rapidly between Days 1 and 13 in cyclic and pregnant ewes and then decreasing to low levels by Day 15 in cyclic ewes and by Day 19 in pregnant ewes [76]. Increases in expression of enJSRV genes in uterine epithelia are highly correlated with changes in circulating levels of progesterone in peripheral blood, but most importantly, are limited to the period when PR are expressed in uterine epithelia. All of these results suggest that one or more enJSRV long terminal repeats (which contains the retroviral promoter and enhancers) are directly regulated by progesterone [76].

Role of enJSRVs in Ovine Pregnancy Recognition

Interferon tau (IFN τ) is a type I interferon, that serves as the critical anti-luteolytic signal necessary for establishment of pregnancy in ruminants [82]. It is produced by the ovine conceptus trophoctoderm between Days 12 and 21 of pregnancy and binds to the type I IFN receptors located on the LE, GE, and stromal cells of the endometrium [102].

Of particular interest is the correlate expression of enJSRV and IFN τ during early pregnancy. Expression of enJSRVs in uterine epithelia of ewes between Days 11 to 19 of pregnancy is temporally correlated with IFN τ production by mononuclear cells of trophoderm [79, 103]. A present hypothesis is that the high levels of enJSRVs in the LE and GE may stimulate trophoblast proliferation and production of IFN τ [79].

Role of enJSRVs Envelope Protein in Ovine Placentation

A key regulatory factor of placental formation may be the enJSRV envelope protein. The rate of proliferation of trophoderm cells during the peri-implantation period suggests that the enJSRV envelope protein may stimulate cell division in a manner akin to the transforming properties induced by the exogenous JSRV envelope protein at the site of infection in the lung [58, 64, 76]. Palmarini et al., [96] demonstrated expression of JSRV capsid and envelope proteins by uterine LE and GE and their presence in binucleate cells of the conceptus trophoderm that forms syncytia with uterine LE. The binucleate cells are formed from mononuclear cells of the trophoblast, which duplicate their DNA without dividing [20]. Only the binucleate cells display invasive properties in the placenta of ruminants as these cells of the developing placenta migrate and fuse with the endometrial LE to form syncytial plaques. The syncytial plaques are necessary for the subsequent formation of placentomes, which are the primary site of nutrition and gas-exchange for fetal growth during gestation as well as a source of hormone production, including placental lactogen and progesterone. As previously discussed, the syncytial plaques are similar to the syncytiotrophoblast of the

human placenta, thus making the sheep an excellent model for investigation of formation and function.

CHAPTER III

SHEEP ENDOGENOUS BETARETROVIRUSES (enJSRVs)

AND THE HYALURONIDASE 2 (HYAL2) RECEPTOR IN THE

OVINE UTERUS AND CONCEPTUS

Introduction

A unique feature of retroviruses is their presence as inherited elements in the germline of most eukaryotes. These elements, known as endogenous retroviruses (ERVs), are transmitted through the germline as stable Mendelian genes [21-23]. It is assumed that ERVs derived from integration events during evolution of ancient exogenous retroviruses (e.g. transmitted horizontally) into the germline of host animal species. The biological relevance of ERVs in mammals has been intensely debated. ERVs have been characterized either as dispensable junk DNA or as indispensable for mammalian development and physiological function [20]. Generally, endogenous proviruses are transcriptionally silent and are often defective, typically differing from the exogenous counterpart by deletions or point mutations that render them incapable of forming infectious virus. However, several ERVs maintain at least some intact open reading frames that can be expressed and are associated with either beneficial or detrimental effects to the host [28, 34-38].

Expression of ERVs in the genital tract and placenta of various animal species has been described for at least three decades [27, 33, 39, 40], sparking opinions on their significance in the evolution of placental mammals and the development of viviparity [29, 39, 43, 104]. In humans and primates, several ERVs, including ERV-3 and HERV-

W, appear to play direct roles in formation of the syncytiotrophoblast of the placenta [26, 29]. The product of the HERV-W envelope (*env*) gene is a highly fusogenic membrane glycoprotein termed syncytin that induces formation of syncytia upon interaction with the type D mammalian retrovirus receptor [6]. Available results support the hypothesis that ERVs have biological functions in placental morphogenesis in humans and other primates.

Sheep represent an interesting model to study the biology of ERVs and their interaction with host species [70, 105-107]. The ovine genome contains approximately 20 copies of endogenous betaretroviruses (enJSRVs) [108] that are highly related to two oncogenic exogenous betaretroviruses, Jaagsiekte sheep retrovirus (JSRV) and Enzootic nasal tumor virus (ENTV) [64, 109]. Hyaluronidase 2 (HYAL2) is a glycosylphosphatidylinositol-anchored cell surface protein with weak hyaluronidase activity that serves as a cellular receptor for JSRV and enJSRV [67, 110]. Interestingly, in vitro assays found that enJSRVs can block JSRV replication at early and late steps of the replication cycle [110], supporting the hypothesis that enJSRVs protect the host during evolution against pathogenic retroviral infections [70].

enJSRVs are transcriptionally active in the fetal and adult sheep and particularly abundant in the female reproductive tract [70, 76, 77]. In the uterus, enJSRVs RNA and protein are expressed exclusively in the endometrial LE and GE [6, 76, 111]. In addition to the endometrium, enJSRVs RNA expression was detected in the trophoblast giant binucleate cells (BNC) of Day 18 and 20 conceptuses, which form the outer layer of the fetal-placental cotyledon and give rise to the syncytial plaques [84, 112].

Trophoblast BNC are thought to arise from the mononuclear trophoctoderm cells (MTC), then migrate through the apical trophoctodermal tight junctions of the chorion, and become apposed to the apical surface of the endometrial LE [84]. Endometrial LE and BNC then fuse apically and form a syncytium of trinucleate cells within the endometrial epithelium. Subsequently, the trinucleate cells enlarge by continued BNC migration and fusion that results in multinucleated syncytial plaques linked by tight junctions [84]. In sheep, the size of the plaques is limited to 20-25 nuclei [86]. The syncytial plaques eventually cover the surface of the endometrial caruncles and aid in development of placentomes, which are formed by interdigitation of fetal placental cotyledons and endometrial caruncles and are necessary for the conceptus to obtain hematotrophic nutrition from the maternal uterus. The trophoblast BNC in the sheep placenta are, in many respects, analogous to the trophoblast giant cells of the syncytiotrophoblast in humans [6, 76, 113].

Little is known about the cellular and molecular mechanisms that regulate trophoblast differentiation and syncytia formation during syneplitheliochorial placentation in sheep. Based on the temporal and spatial alterations in enJSRVs expression in the ovine uterus and placenta, we have hypothesized that the enJSRVs have biological roles in protection of the uterus against viral infection and in affecting placental morphogenesis [77]. Specific objectives of this study were to: (i) determine the ontogeny and expression patterns of enJSRVs *env* and *HYAL2* mRNA during gestation in ovine uteroplacental tissues; and (ii) determine which enJSRVs loci are

transcriptionally active in uteroplacental tissues by sequencing and analysis of enJSRVs *env* mRNA expressed in uteroplacental tissues.

Materials and Methods

Animals and Experimental Design

Mature ewes of primarily Suffolk breeding were observed daily for estrus (designated Day 0) using vasectomized rams. All ewes exhibited at least two estrous cycles of normal duration (~16 to 18 days). At estrus, ewes were bred to intact rams at 12 h and 24 h post-estrus. All experimental and surgical procedures involving animals met the Guidelines for the Care and Use of Agricultural Animals in Agricultural Teaching and Research and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

In Study One, pregnant ewes were assigned randomly to be ovariectomized (n= 4 ewes/day) on either Day 20, 30, 40, 50, 60, 80, 100, or 120 of pregnancy (Day 0 = mating). At hysterectomy, the uterus was trimmed free of cervix and oviduct and opened along the mesometrial border. Several sections (~0.5 cm) of both intercaruncular and placentomal regions from the mid-portion of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). At hysterectomy, remaining placentomes were removed by physical dissection and additional intercaruncular endometrium was dissected from the myometrium. Various placentomes were physically separated into maternal endometrial caruncle and fetal placental cotyledon. All collected samples of

uteroplacental tissues were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

In Study Two, cyclic ewes were mated at estrus (Day 0) and Day 1 to fertile rams. At mating, the ewes were assigned randomly to have the conceptus (embryo/fetus and associated extraembryonic membranes) recovered by flushing the uterine lumen with phosphate buffered saline on either Day 10, 12, 14, 16, or 18 post-mating (n=5 conceptuses/day). The collected conceptuses were snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

RNA Extraction

Total cellular RNA was isolated from tissues using Trizol (Gibco-BRL, Bethesda, MD) according to manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and integrity of the RNA was examined following gel electrophoresis in a denaturing 1% agarose gel.

In situ Hybridization Analysis

The enJSRV *env* and *HYAL2* mRNAs were localized in uterine tissue sections (5µm) by in situ hybridization analysis as described previously [63]. The ovine *HYAL2* cDNA was kindly provided by Dr. Michael Lerman (National Cancer Institute, Frederick, MD) [77]. Deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized ovine endometrial enJSRV *env* cDNA (DD54) [77] or *HYAL2* cDNA by in vitro transcription with [α -³⁵S]UTP. After hybridization, washing, and RNase A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak,

Rochester, NY), stored at 4°C for 1 week, and developed in Kodak D-19 developer. Slides were then counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated through a graded series of alcohol to xylene, and protected with a coverslip.

Northern Blot Analysis

Intercaruncular endometrial or placentomal total RNA was denatured, separated using a 1.5% agarose denaturing gel, and then transferred onto a Nytran Plus positively charged nylon membrane (Schleicher & Schuell, Inc.) by downward blotting as described previously [114]. Radiolabeled antisense complementary enJSRV *env* (DD54) RNA probes were then generated from linearized plasmid DNA templates by in vitro transcription with [³²P- α]UTP and either SP6 or T7 bacterial RNA polymerases using a MAXIscript SP6/T7 kit (Ambion, Inc.). Membranes were hybridized with radiolabeled antisense cRNA probes and washed as described previously [115]. After digestion and washing, autoradiographs were produced using X-Omat AR film (Kodak) and cassettes with intensifying screens.

RT-PCR Analysis

Expression of enJSRVs RNA and the ovine Hyal2 receptor were determined by RT-PCR using methods described previously [108]. Briefly, cDNA was synthesized from total endometrial, placentomal or conceptus RNA (5 μ g) using random and oligo-dT primers and SuperScript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 μ l sterile water, and stored at -20°C. The cDNAs were diluted (1:10) with sterile water

prior to use in PCR reactions. The PCR reactions were performed using AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) and Optimized Buffer H (Invitrogen, Carlsbad, CA) according to manufacturers' recommendations.

PCR primers from sequences flanking the carboxy-terminal portion of the transmembrane (TM) domain of the JSRV Env region were derived from the sequence of the JSRV₂₁ infectious molecular clone [63] (GenBank # AF105220), because this region is known to be well conserved among exogenous and endogenous sheep betaretroviruses. The enJSRV *env* primers, enJSRVenvF (5' – AACATTTGCAAGGAATTTGG-3') and enJSRVenvR (5'-GCTCCATAAGATGTTGGTGC-3') amplified a cDNA of 336 bp. The enJSRV *env* PCR amplifications were conducted as follows: 35 cycles at 95°C for 30 sec; 53.5°C for 1 min; and 72°C for 1 min. The *HYAL2* primers, bLuca2F135 (5'-CCAGCATGTGGACAGGCCTG-3') and bLuca2R600 (5'-TACACATCCTTGTCCTGCCAG-3'), were derived from the *Ovis aries HYAL2* mRNA (GenBank AF411974) and amplified a cDNA of 465 bp as described previously [77]. The *HYAL2* PCR amplifications were conducted as follows: 35 cycles at 95°C for 30 sec; 60°C for 1 min; and 72°C for 1 min. As a positive control, β -actin primers (forward: 5'-ATGAAGATCCTCACGGAACG-3'; reverse: 5'-GAAGGTGGTCTCGTGAATGC-3') were used to amplify a 270 bp cDNA. The PCR conditions and amount of template cDNA used in each reaction were optimized for each primer set to ensure linear amplification of the target. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining using an Alpha Innotech imaging system.

Isolation and Sequence Analysis of enJSRV Env Clones

To determine the identity of uterine enJSRVs Env, a Day 14 pregnant ovine endometrial cDNA library, constructed by Clontech (Palo Alto, CA, USA) using directional cloning into the pTriplEx2 phage vector, was screened using the radiolabeled antisense ovine enJSRV *env* cRNA probe (DD54 that corresponds to a portion of enJSRVs *env*) [116]. Five clones, purified by three sequential hybridization screenings, were then excised from the lambda-TriplEx2 phage using BM25.8 cells for subsequent transformation into DH5 α cells prior to cDNA extraction (Qiagen, Valencia, CA) and sequencing in both directions using an ABI 373XL DNA sequencer (Applied Biosystems, Foster City, CA) and pTriplex sequencing primers (Clontech, Palo Alto, CA, USA). Nucleic acid similarity searches were performed using the basic local alignment and search tools (BLAST) at the National Center for Biotechnology Information (NIH, Bethesda, MD) [110]. Nucleotide sequence structure analyses were performed using the BCM search launcher (Human Genome Sequence Center, Baylor College of Medicine, Houston, TX).

To determine the identity of enJSRV loci that were transcriptionally active in the endometrium, placenta, and placentome (cotyledon and caruncle), RT-PCR analysis was conducted using the enJSRV *env* primers listed previously and the methods previously described [77, 117]. Tissues from Day 120 pregnant ewes (n=3) were used for RT-PCR. PCR products were cloned into pCR2.1 (Invitrogen), and eight independent clones were analyzed in each tissue type evaluated. Similarity searches were conducted as previously described, with ClustalW alignment and phylogenetic tree assembly

conducted utilizing the alignment services of the European Bioinformatics Institute (EMBL-EBI) available online at <http://www.ebi.ac.uk/services>.

Results

Expression of enJSRV RNAs in the Uterus of Pregnant Ewes

RT-PCR analysis using enJSRV *env* specific primers allowed the amplification of a 336 bp cDNA product that sequence analysis confirmed to have originated from enJSRVs *env* (data not shown). In the conceptus, enJSRVs RNA was expressed between Days 10 and 12 (Figure 3.1A). enJSRVs *env* mRNA was also amplified in both the intercaruncular endometrium and placentomes, regardless of day of gestation (Figures 3.1B and 3.1C). As an internal positive control, the β -actin mRNA was amplified in all conceptus, endometrium and placentome samples (Figure 3.1D).

Additional information regarding the temporal expression of enJSRVs is illustrated via Northern blot analyses of total RNA isolated from endometria and placentomes. As expected, by using a cRNA enJSRVs *env* probe, Northern blot analyses detected both the full-length enJSRV genomic DNA (7.5 kb), as well as the correctly spliced enJSRV *env* mRNA (2.4 kb) [63] in intercaruncular endometria from Days 12 to 30 (Figure 3.1E). The 2.4 kb enJSRV *env* mRNA was predominantly detected in the intercaruncular endometrium after Day 30 and in placentomes from Days 40 to 120 of gestation.

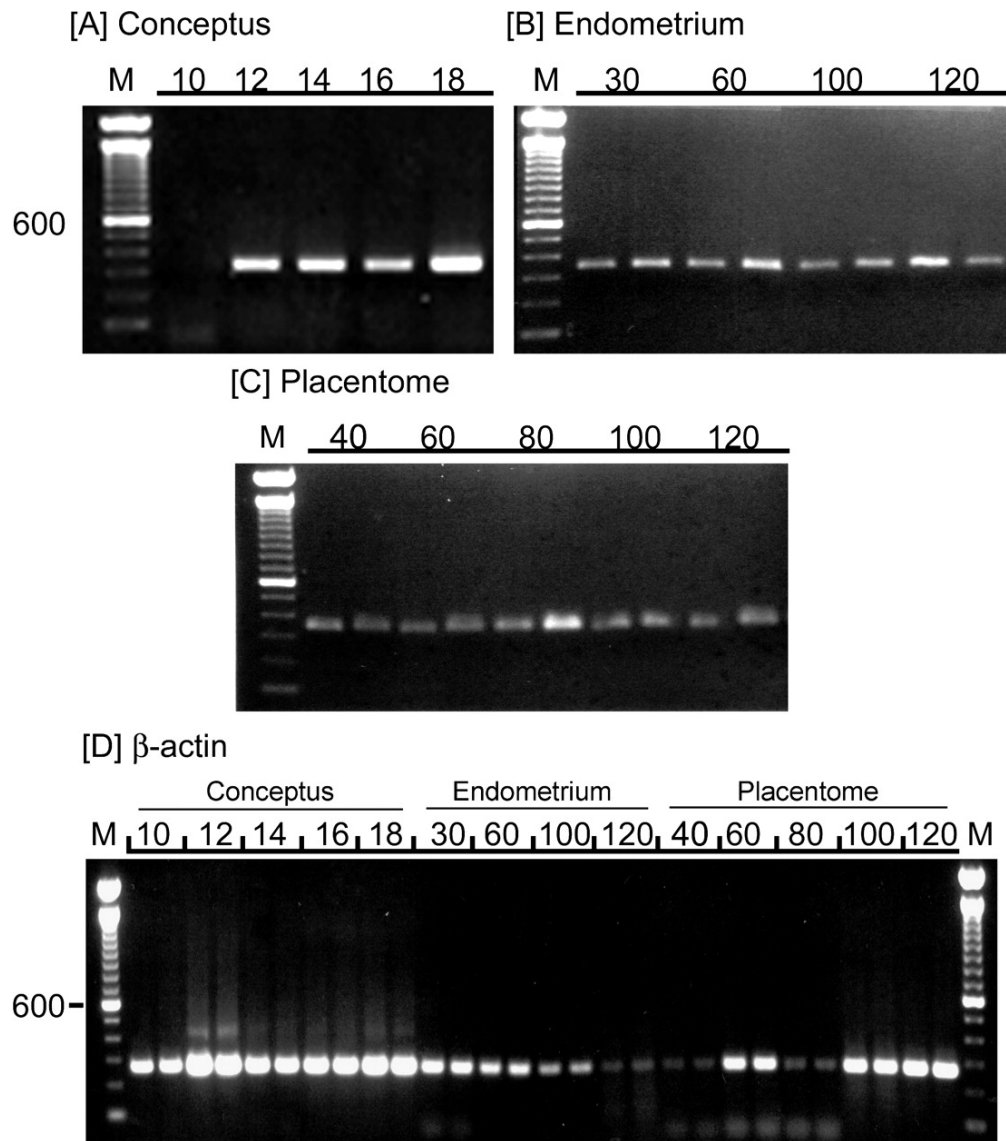
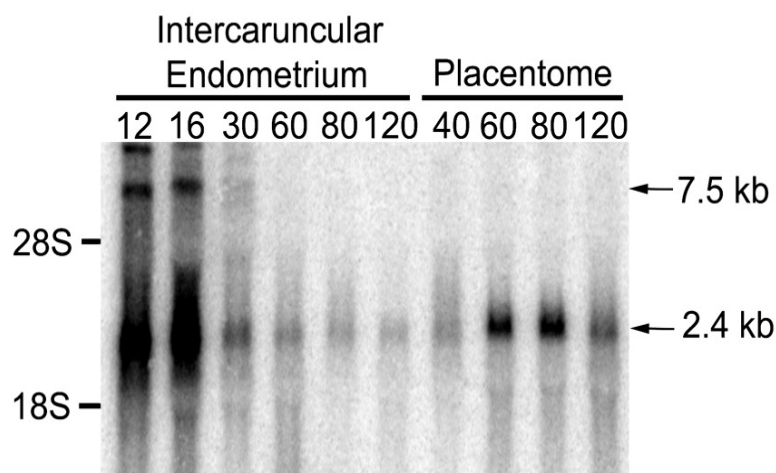


Figure 3.1 Expression of enJSRVs *env* mRNA in ovine uteroplacental tissues. [A, B, and C] Representative RT-PCR analysis of enJSRVs *env* mRNA in the [A] early conceptus, [B] endometrium, and [C] placentomes from pregnant ewes. PCR products were separated in a 1.5% agarose gel and visualized using ethidium bromide. The 100 bp marker (M) is shown on the left portion of each gel. [D] β -actin amplification in all samples.

[E] Northern blot analysis

**Figure 3.1 Continued.**

[E] Northern blot analysis of total RNA from the intercaruncular endometrium and placentome from pregnant ewes. Endometrial total RNA (20 μ g) was separated on a 1.2% denaturing agarose gel, transferred to a nylon membrane and hybridized with radiolabeled antisense enJSRV *env* cRNA probe (DD54). Positions of the 28S and 18S rRNAs are indicated on the left. The 7.4 kb RNA corresponds to the full-length enJSRV genome, and the 2.4 kb mRNA likely represents the correctly spliced *env* RNA.

In situ hybridization analyses revealed that in the intercaruncular endometrial tissues enJSRVs RNA was expressed exclusively in the endometrial LE and superficial GE from all ewes regardless of gestational day (Figure 3.2). Expression of enJSRVs RNA was very low in the middle to deeper endometrial GE. Expression of the enJSRV RNAs was abundant in the cotyledonary portion of the placentomes (Figure 3.3). The enJSRV RNAs were specifically expressed in trophoblast giant BNC and syncytial plaques that form the outer lining of the cotyledonary chorionic villus of the placenta. However, expression of enJSRV RNAs was not detected in the caruncular portion of placentome.

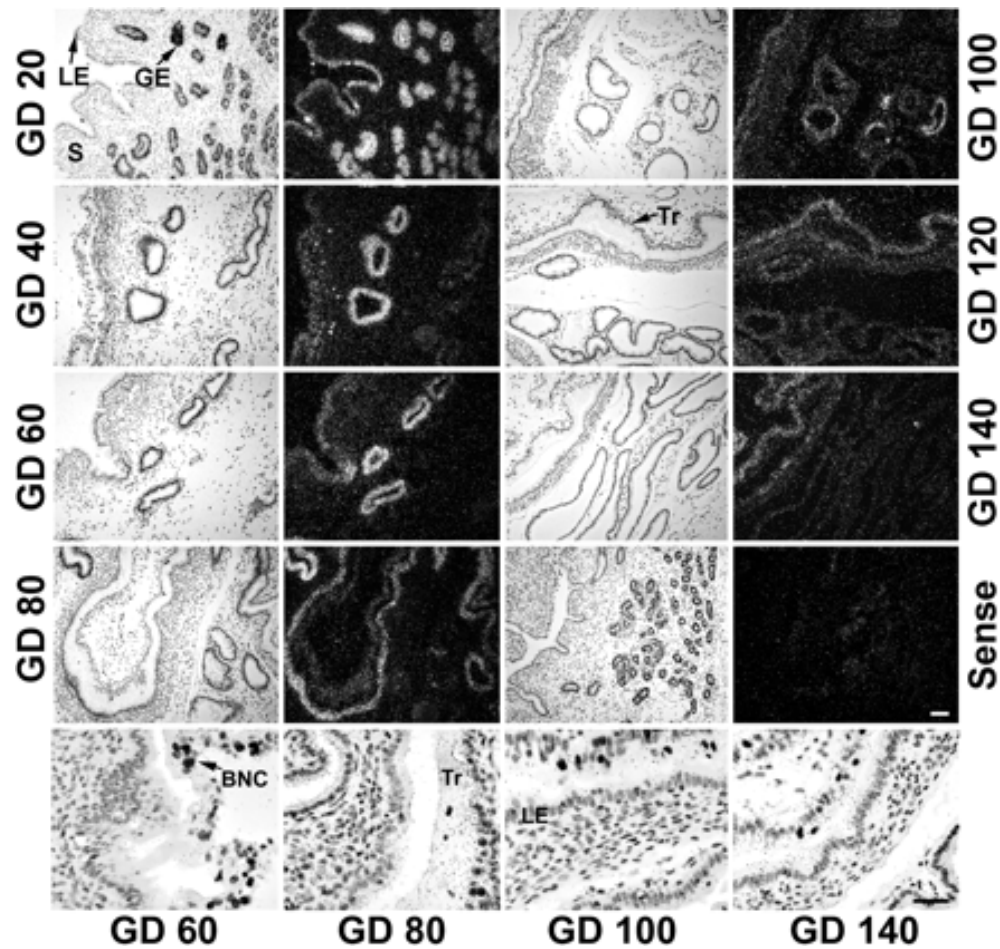


Figure 3.2. *In situ* hybridization analysis of enJSRVs RNA expression in the intercaruncular endometrium from pregnant ewes. Cross-sections of the intercaruncular endometrium and placentomes of pregnant ewes were hybridized with radiolabeled antisense or sense ovine enJSRV *env* cRNA probes. Protected transcripts were visualized by liquid emulsion autoradiography for one week and imaged under brightfield or darkfield illumination. Legend: BNC, binucleate cell; GD, gestational day; LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophoblast. Scale bar = 20 μ m.

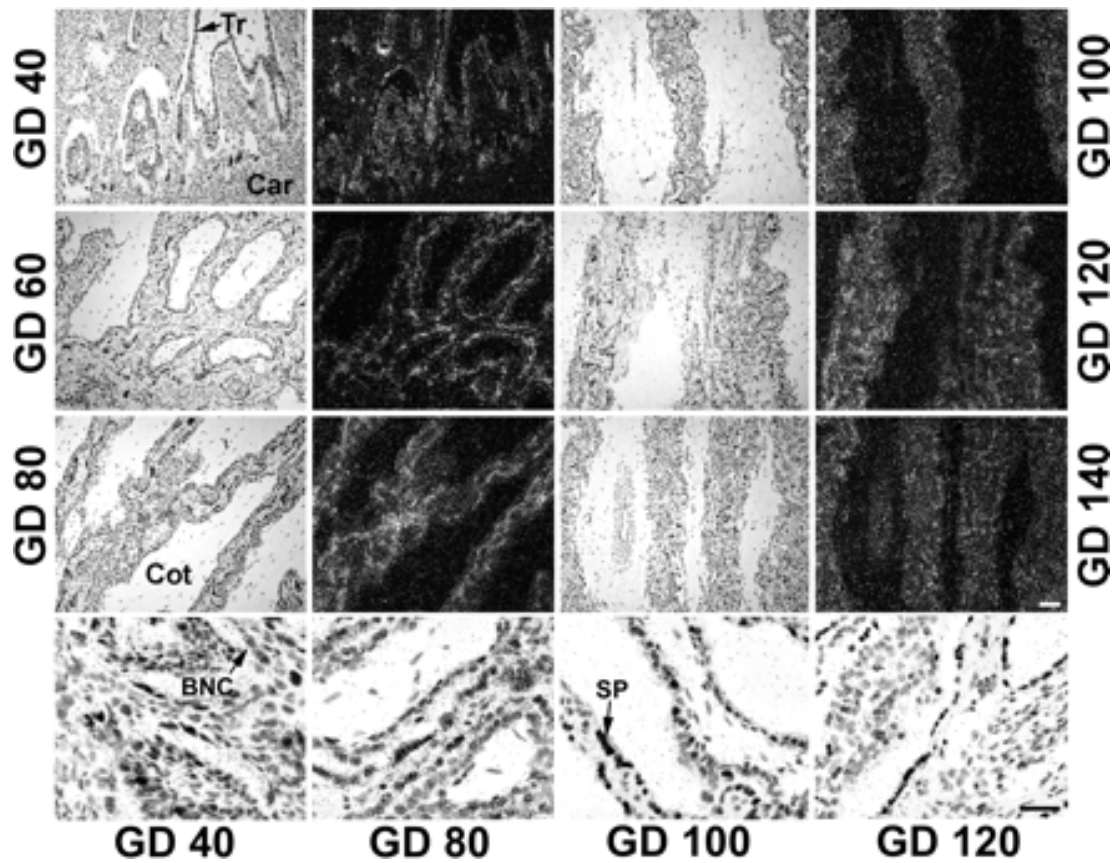


Figure 3.3. *In situ* hybridization analysis of enJSRV RNA expression in the placentomes from pregnant ewes. Cross-sections of the intercaruncular endometrium and placentomes of pregnant ewes were hybridized with radiolabeled antisense or sense ovine enJSRV *env* cRNA probes. Protected transcripts were visualized by liquid emulsion autoradiography for one week and imaged under brightfield or darkfield illumination. Legend: BNC, binucleate cell; Car, endometrial caruncle; Cot, placental cotyledon; GD, gestational day; SP, multinucleate syncytial plaque; Tr, trophoctoderm. Scale bar = 20 μ m.

Expression of the enJSRV receptor *HYAL2* in the Uterus of Pregnant Ewes

HYAL2 mRNA expression in the conceptus, intercaruncular endometrium, and placentome was determined by RT-PCR analysis of total RNA using ovine *HYAL2* specific primers (Figure 3.4). The 465 bp cDNA RT-PCR product was cloned and sequenced to confirm its identity as *HYAL2* (data not shown). In the conceptus, *HYAL2* mRNA was not detected on Days 10, 12, or 14, but was evident in the Day 16 and 18 conceptuses (Figure 3.4A). Total RNA from sheep lung was used as a positive control for *HYAL2* [71]. *HYAL2* mRNA was not detected in the intercaruncular endometrium, regardless of day of gestation (Figure 3.4B). In contrast to the intercaruncular endometrium, *HYAL2* mRNA was detected in placentomes from Days 40 to 120 of gestation (Figure 3.4C). The RNA in samples not positive for *HYAL2* mRNA was not degraded, because β -actin, a housekeeping gene, could be amplified in all conceptus and endometrium samples (Figure 3.1D).

In situ hybridization analysis revealed that *HYAL2* mRNA was expressed exclusively by the BNC and syncytial plaques of the placental cotyledons, but not in other cell types in the placentome (Figure 3.5). As expected from the RT-PCR analyses,

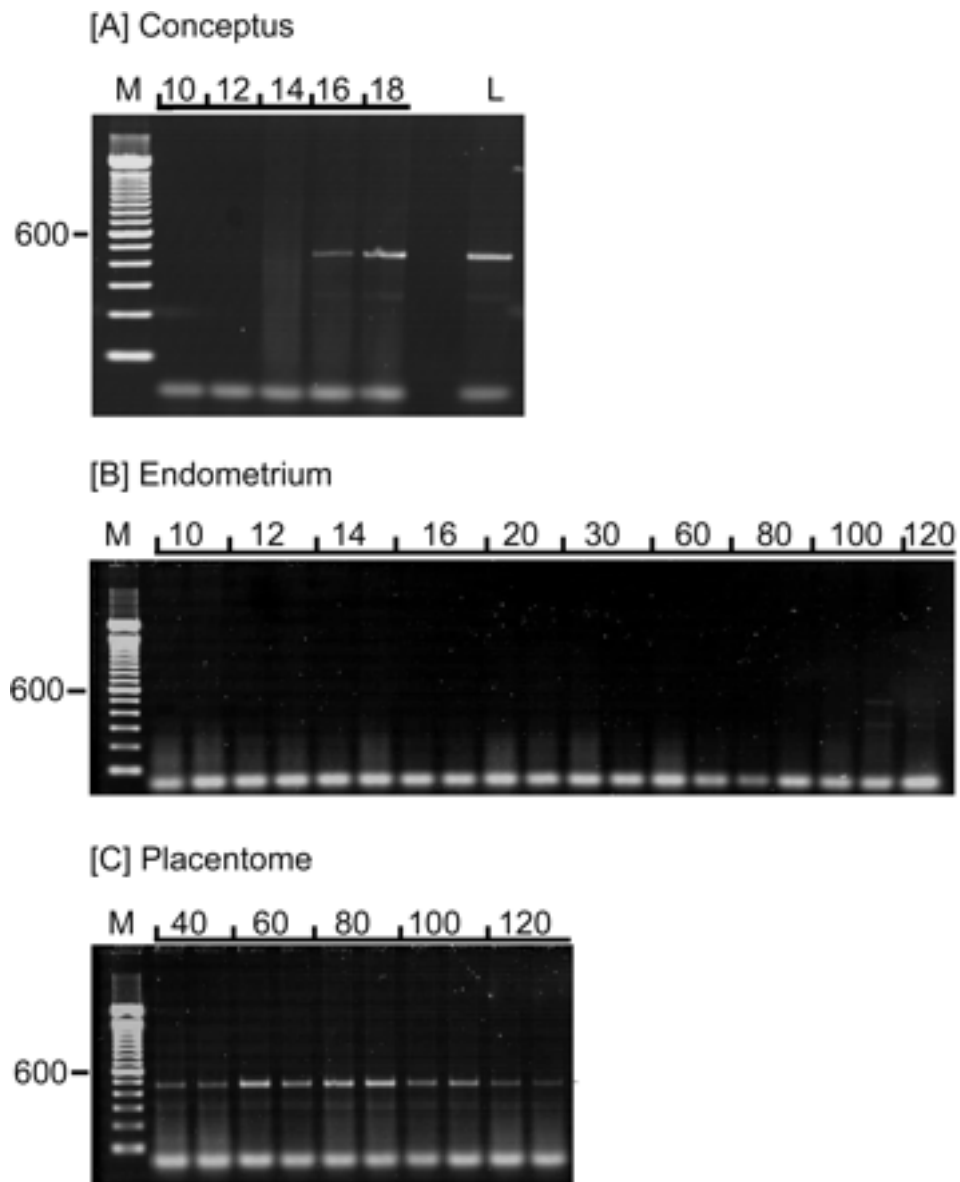


Figure 3.4 Expression of *HYAL2* mRNA in ovine uteroplacental tissues. [A, B, and C] Representative RT-PCR analysis of *HYAL2* mRNA in the (A) early conceptus, (B) endometrium, and (C) placentomes from pregnant ewes. The lung (L) was used as a positive control. PCR products were separated in a 1.5% agarose gel and visualized using ethidium bromide. The 100 bp marker (M) is shown on the left portion of each gel.

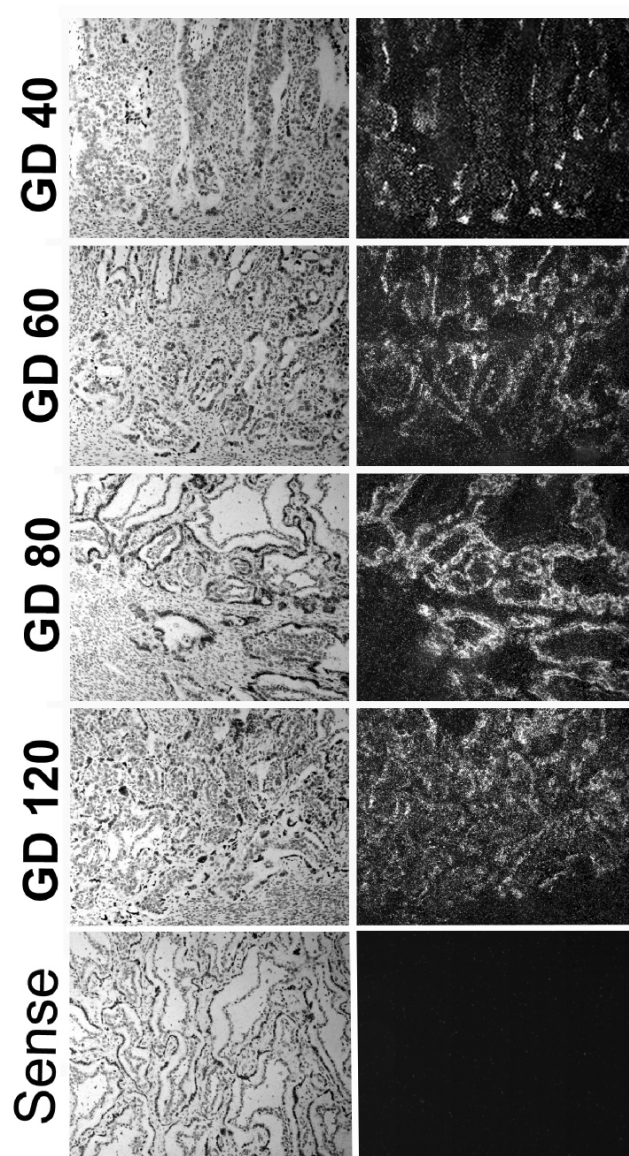


Figure 3.5. *In situ* hybridization analysis of *HYAL2* RNA expression in the placentomes from pregnant ewes. Cross-sections of the placentomes of pregnant ewes were hybridized with radiolabeled antisense or sense ovine *HYAL2* cRNA probes. Protected transcripts visualized by liquid emulsion autoradiography for one week and imaged under brightfield or darkfield illumination. Legend: Car, endometrial caruncle; Cot, placental cotyledon; GD, gestational day; SP, multinucleate syncytial plaque. Scale bar = 20 μ m.

HYAL2 mRNA was not expressed in the intercaruncular endometrium or myometrium (data not shown).

Cloning and Analysis of enJSRVs *env* RNAs Expressed in the Uteroplacenta

There are approximately 20 copies of enJSRVs in the sheep genome, but no information is available on which loci are expressed in the uterus and placenta. As a first step to identify the transcriptionally active enJSRVs, five 1.4-kilobase (kb) cDNAs encoding enJSRV *env* mRNAs were isolated by screening a Day 14 pregnant ovine endometrial cDNA library with an enJSRV *env* probe. All five plaque purified clones were full-length with an intact open reading frame. The inferred amino acid sequences were 94-99% identical to each other, 95-99% to enJS56A1 and enJS5F16 Env, and 89-90% identical to the exogenous JSRV Env (Figure 3.6). All of the enJSRV *env* clones lacked the YXXM motif present in the cytoplasmic tail of the TM domain of the JSRV Env reported to be critical for transformation [70]. This deleted region is a feature conserved amongst all the Env sequences of the enJSRVs.

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enJS56A1  MPKRRAGFRKGWYARQRNSLTHQMQRMTLSEPTSELPTQRQIEALMRYAWNEAHVQPPVPTPTNILIMLLLLLQRIQNGAAAAFWAYIP
enJS5F16  .....HK.....
enJSRV_13 .....Q.....C.....
enJSRV_12 .....Q.....C.....
enJSRV_14 .....Q.....C.....
enJSRV_15 .....Q.....C.....
enJSRV_16 .....Q.....C.....
JSRV21    .....V.....

enJS56A1  FMIQSLGWDKETVPVYVNDTSLGKGSDIHISPPQANISFYGLTTQYPMCFYQSQHPHCQVSADISYPRVTISGIDEKTGKRSYRD
enJS5F16  .....
enJSRV_13 .....
enJSRV_12 .....S.....
enJSRV_14 .....
enJSRV_15 .....L.....F.....
enJSRV_16 .....Y.....
JSRV21    .....R.I.....K..GN

enJS56A1  GPLDIPFCDKHLISIGIGIDTPWTLCRAPIASVYNINNANTLLWDWAPGGTPDFPEYRGQHPPIFSVNTAPIYQTELWKLAAFGHGN
enJS5F16  .....R.....L.....F.....
enJSRV_13 .....N.....R.....L.....F.....
enJSRV_12 .....R.....L.....F.....
enJSRV_14 .....N.....R.....L.....F.....
enJSRV_15 .....N.....R.....L.....F.....
enJSRV_16 .....R.....L.....F.....
JSRV21    .....RV.....A.F.....

enJS56A1  YLQPNISGSKYGDVGVTGFLYPRACVPYPFMLIQGHMEITLSLNIYHLNCSNCILTNCIRGVAKGEQVIIVKQPAFVMLPVEITEEWY
enJS5F16  .....
enJSRV_13 .....PP...L.K..I.N...Y.....KV.....I.....
enJSRV_12 .....
enJSRV_14 .....PP...L.K..I.N...Y.....KV.....I.....
enJSRV_15 .....PP...L.K..I.N...Y.....KV.....I.....
enJSRV_16 .....
JSRV21    .....A.A..

SU ↔ TM

enJS56A1  TALELLQRINTALSRPKRGLSLIILGIVSLITLIATAVTASVSLAQSIQAHTVDSLSSNVTKVMGTQENIDKKIEDRLSALYDVVRV
enJS5F16  .....Y.....D.....
enJSRV_13 .....L.....Y.....D.....
enJSRV_12 .....Y.....D.....G.....
enJSRV_14 .....L.....Y.....D.....
enJSRV_15 .....L.....Y.....D.....
enJSRV_16 .....Y.....D.....
JSRV21    .....Y.....D.....

enJS56A1  EQVQSINFRMKIQCHANYKWICVTKKPYNTSDFPWDKVKKHLQGIWFNTTVSLDLLQLHNEILDIENSPKATLNIADTVDNFLQNLF
enJS5F16  .....H.....N.....
enJSRV_13 .....N.....N.....
enJSRV_12 .....N.....M.....F.....L.....N.F.NF...I...FC.KIFH.....VVP.I.....
enJSRV_14 .....N.....N.....
enJSRV_15 .....N.....N.....
enJSRV_16 .....H.....N.....
JSRV21    .....NL.....

enJS56A1  PSLHSLWRSIIAMGAVLTVVLIICLAPCLIRSIVKEFLHMRV-LIH---KNMLQHQLMELLKNKERGAAGDDP 611
enJS5F16  .....Q.....-...-...R..... 611
enJSRV_13 .....Q.....-...-...R..... 611
enJSRV_12 .....Q.....-...-...R..... 611
enJSRV_14 .....Q.....-...-...R.....L 611
enJSRV_15 .....Q.....-...-...R.....AL 611
enJSRV_16 .....Q.....-...-...R..... 611
JSRV21    .....KTL.GV.ILVFIIIVV.LIF...V.GM.RD..K...EML.MKYR.....D..... 615

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VR3

Figure 3.6. ClustalW analysis of protein sequences from enJSRVs *env* cDNAs isolated from a Day 14 pregnant endometrial cDNA library. Alignment includes comparison to the known enJSRVs (enJS5F16 and enJS56A1) and the exogenous JSRV (JSRV21). A period represents a conserved substitution; and dashed lines represent deletions. The superficial (SU) and transmembrane (TM) areas and variable region 3 (VR3) is denoted in the figure.

In order to determine whether the transcriptionally active enJSRV loci followed a tissue-specific pattern of expression, RT-PCR was used to clone partial enJSRV *env* cDNAs from endometrium, placenta, caruncles and cotyledons from Day 120 pregnant ewes. The primers used for RT-PCR were designed to amplify a region of the C-terminus of the JSRV Env containing the variable region 3 (VR3) that is lacking in the enJS5F16 and enJS56A1 *env* genes [118]. Sequence analysis revealed that the clones derived from the pregnant ovine tissues were more than 95% identical to enJS5F16 and enJS56A1. All of the enJSRVs *env* sequences maintained an open reading frame and lacked the YXXM motif that is present in the VR3 region of the TM domain of the exogenous JSRV Env. We obtained 20 unique RT-PCR clones, suggesting that most of the 20 predicted enJSRV loci are transcriptionally active in the endometrium. Results of phylogenetic analysis of the partial enJSRVs Env is presented in Figure 3.7.

All of the partial enJSRV Env sequences were aligned and examined for rates of synonymous (dS) and nonsynonymous (dN) substitutions using the method of Nei and Gojobori [119] incorporating a statistic from Ota and Nei [120] implemented in the SNAP program [118]. The sequences alone gave a dN/dS ratio of 0.33. A dN/dS < 1 is

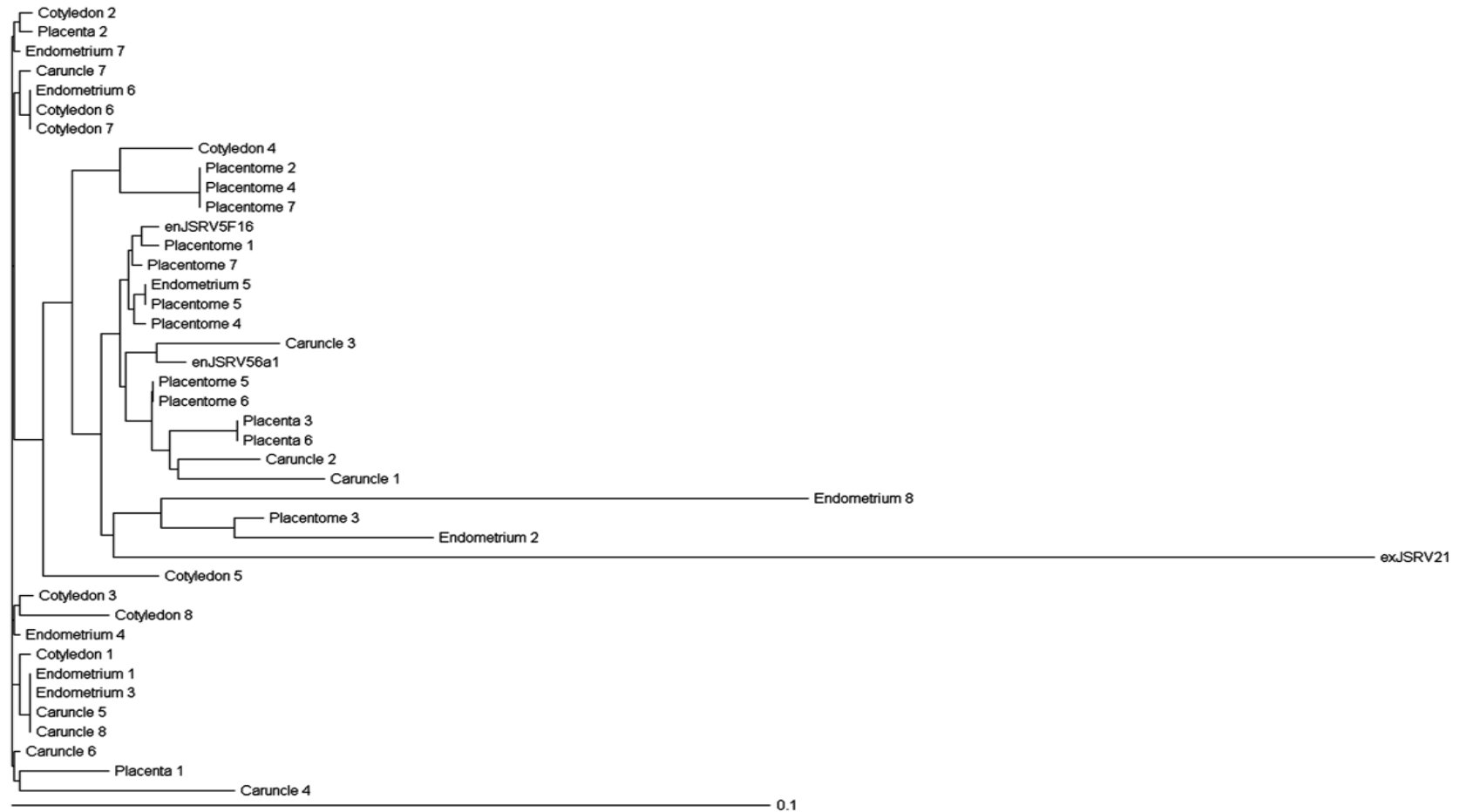


Figure 3.7. Phylogenetic analysis of enJSRVs. Phylogenetic tree based on the Neighbor Joining method using 10,000 bootstraps within the partial enJSRV *env* cDNAs cloned by RT-PCR from uteroplacental tissues and known sequences of enJSF16, enJS56A1, and JSRV21 Env. The branch lengths are proportional to an estimate of evolutionary change.

evidence of purifying selection [121], suggesting that enJSRVs appear to be subject to purifying selection, and have functional involvement in the ovine uterus and placenta.

Discussion

The analysis of enJSRVs *env* isolated from cDNA library screening and RT-PCR analysis of uteroplacental tissues revealed sequences highly homologous to the previously described enJSRVs Env. No tissue-specific expression of any particular locus was observed in the endometrium, placentome or placenta. Given the heterogeneity of the sequences obtained by RT-PCR cloning, it appears that most, if not all, of the 20 predicted enJSRV loci are transcriptionally active in the ovine uterus and placenta and encode Env with an open reading frame. The analysis of cloned partial enJSRV Env sequences based on rates of synonymous (dS) and nonsynonymous (dN) substitutions provided evidence of purifying selection, which supports our working hypothesis that the enJSRVs have important biological roles in uterine function and placental morphogenesis. Interestingly, Bonnaud and coworkers [67, 76] recently found conserved selective constraints on the *env* of ERVWE1 that encodes syncytin in humans and primates, suggesting that this retroviral locus has been recruited in the hominoid lineage to become a bona fide gene.

In previous studies we determined that enJSRVs were expressed in uteri from neonatal and adult cyclic and early pregnant ewes [81, 84, 85]. In the present study, enJSRV RNAs were found to be expressed throughout gestation in the uterus and specifically in the endometrial LE and GE of the intercaruncular areas. Indeed, enJSRVs *env* and *HYAL2* mRNAs were first detected by RT-PCR in Day 12 and Day 16

conceptuses, respectively, which coincides with the initial differentiation of the BNC from the MTC that begins between Days 14 to 16 [39, 43]. In the placenta, enJSRV *env* mRNA was specifically expressed in the trophoblast giant BNC and multinucleated syncytial plaques. *HYAL2* mRNA was also detected specifically in the BNC and syncytial plaques throughout gestation. These results combined with those from studies of human ERVs [67, 110] and enJSRVs [6, 67, 110] provide strong support for the hypothesis that enJSRVs have beneficial biological roles for the host including: (1) protection from exogenous infectious viruses; (2) local suppression of immune recognition of the conceptus; and (3) placental morphogenesis.

The enJSRVs expressed in the endometrial LE and GE may have protected the uterus from infection by exogenous and pathogenic infectious betaretroviruses during evolution. This hypothesis is supported by recent evidence that expression of enJSRVs blocked entry and exit of the exogenous JSRV by receptor interference and the action of a transdominant Gag protein of the enJSRV locus enJS56A1 [33]. In the present study, cloning of the expressed enJSRVs *env* mRNA from ovine uteroplacental tissues revealed sequences that were similar, but not identical, to the previously identified enJS5F16 and enJS56A1 loci. Thus, all of the 20 enJSRV loci need to be cloned and functionally analyzed for their ability to interfere with JSRV entry and replication through known or novel mechanisms.

The Metavirus hypothesis states that all mammals must express immunosuppressive ERV proteins in extraembryonic tissues to suppress local immune recognition of the embryo [85]. Sheep and other domestic ruminants exhibit a

synepitheliochorial type of placentation in which the endometrial LE persists but is modified to a variable degree, depending on species, into a hybrid fetomaternal syncytium formed by the migration and fusion of BNC with LE, which is functionally equivalent to the syncytiotrophoblast layer of the human placenta [6, 67]. Sheep affected by ovine pulmonary adenocarcinoma (OPA) or ENTV do not show an appreciable antibody response to JSRV or ENTV which supports the hypothesis that enJSRV expression in the fetal lamb tolerizes sheep to the related exogenous viruses, JSRV and ENTV [39]. However, the potential immunological consequences of enJSRVs have not been investigated as proposed for several human ERVs [84, 85].

The enJSRVs Env is also hypothesized to have a biological role in placental morphogenesis. The timing and localization of enJSRVs expression support the possibility that enJSRVs play a role in MTC differentiation into BNC as well as BNC fusion with the endometrial LE to form syncytial plaques. For instance, expression of enJSRVs and HYAL2 in the elongating peri-implantation conceptus coincides with initial differentiation and formation of trophoblast giant BNC. The enJSRVs are expressed throughout pregnancy in the BNC and syncytial plaques of the cotyledonary portion of the placentomes. Indeed, the BNC are thought to continue to differentiate, migrate and fuse to form multinucleated syncytia throughout most of pregnancy in sheep [27, 39, 43]. Studies of ERVs in the human placenta [26, 29, 104] provide substantial support for this hypothesis. In humans, the product of the HERV-W *env* gene is a highly fusogenic membrane glycoprotein termed syncytin that induces trophoblast cell fusion, differentiation and formation of syncytia on interaction with the type D mammalian

retrovirus receptor [67]. The enJSRVs Env can utilize HYAL2 as a receptor [122], but the ability of the different enJSRVs Env to induce cell fusion and the formation of syncytia has not been reported.

Although the morphological aspects of BNC differentiation are well documented, the cellular and molecular mechanisms regulating their differentiation and development are not understood. All enJSRVs Env expressed in uteroplacental tissues lacked the YXXM motif that is present in the VR3 region of the JSRV Env and that is important for transformation in vitro of rodent fibroblasts [57, 71, 122-124]. Transformation of cells by the Env gene of JSRV can be Hyal2 receptor-independent and involve the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway [124]. Interestingly, activation of the PI3-K/Akt signaling pathway regulates development of the differentiated trophoblast giant cell phenotype in other mammals [30, 31, 125].

Expression of enJSRVs in the BNC and syncytial plaques may influence expression of other genes due to the transcriptional regulatory properties of their long terminal repeats (LTRs) and/or function(s) of the Env protein. For example, a HERV-E insertion in the 5'-untranslated region of the growth factor pleiotrophin (PTN) gene creates a trophoblast-specific promoter [126]. The placenta-specific expression of the insulin-like growth factor INSL4 also appears to be driven by a HERV insertion [127]. Therefore, one may speculate that enJSRVs are important regulators of BNC-specific genes, such as placental lactogen [128] and pregnancy-associated glycoproteins [20], in the ovine placenta.

In summary, the temporal and spatial alterations in expression of enJSRVs and HYAL2 in the sheep uterus and placenta suggest a variety of physiological roles in conceptus implantation and placentation. The sequence and functionality of the candidate enJSRVs identified in the present study will be useful in efforts to unravel their physiological roles in the ovine uterus and placenta.

CHAPTER IV

PROGESTERONE REGULATES EXPRESSION OF SHEEP ENDOGENOUS BETARETROVIRUSES IN THE OVINE UTERUS

Introduction

A unique feature of retroviruses is their presence as inherited elements in the germline of most eukaryotes. These elements, known as endogenous retroviruses (ERVs), are transmitted through the germline as stable Mendelian genes, yet they exhibit structural and sequence similarities to infectious exogenous retroviruses [21, 23, 129]. It is assumed that ERVs derive from integration events during evolution of ancient exogenous retroviruses (e.g. transmitted horizontally) into the germline of host animal species. In recent years, considerable effort has been directed toward understanding the biological significance of ERVs, particularly those present in the human germline [20]. Generally, endogenous proviruses are transcriptionally silent and are often defective, typically differing from the exogenous counterpart by deletions or point mutations that render them incapable of forming infectious virus. However, several ERVs maintain at least some intact open reading frames with expression associated with either beneficial or detrimental effects to the host [25, 26, 28-33]. Specific expression of some ERVs in the placenta has led to various hypotheses that these elements play a role in mammalian reproduction [69, 70, 105, 106, 130].

Sheep represent a unique model to study the biology of ERVs and their interaction with host species. The ovine genome contains 15 to 20 copies of endogenous retroviruses (enJSRVs) [60, 108] that are highly related to two oncogenic exogenous

betaretroviruses, Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV) [76, 77]. Endogenous JSRV RNAs are highly expressed in the uterine epithelia [74, 131, 132] while the exogenous pathogenic viruses, JSRV and ENTV, appear to have a strict tropism for secretory cells of the respiratory tract [77]. Expression of enJSRV RNAs in the ovine uterus was initially identified by differential display PCR and PCR-based subtraction hybridization experiments [76]. In situ hybridization analyses displayed that enJSRV RNAs were restricted in expression to the endometrial luminal epithelium (LE) and glandular epithelium (GE) [67, 70, 77]. Indeed, the relative expression level of the enJSRVs in the uterine endometrial epithelia is very high relative to a number of other genes expressed in the same epithelia as well as expression of enJSRVs in other sheep tissues [115]. We hypothesized that expression of enJSRVs in the endometrial epithelia of the ovine uterus regulate conceptus-endometrial interactions as well as placental morphogenesis. To further investigate the regulation of enJSRVs in sheep uterine biology, studies were conducted to determine the effects of day of pregnancy, progesterone, and interferon tau on expression of enJSRVs in the ovine uterus.

Materials and Methods

Animals and Experimental Design

Mature ewes of primarily Rambouillet or Suffolk breeding were observed daily for estrus using vasectomized rams. All ewes exhibited at least two estrous cycles of normal duration (~16 to 18 days). Experimental and surgical procedures involving animals were approved by the Institutional Agricultural Animal Care and Use

Committee of Texas A&M University. At estrus, ewes were assigned randomly to cyclic or pregnant status. Ewes assigned to pregnant status were bred to intact rams at estrus and at 12h and 24h after detection of estrus.

Fifty-two ewes were assigned randomly to be ovariectomized (n= 4 ewes/day) on either Days 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 80, 100, or 120 of pregnancy (Day 0 = mating). At hysterectomy, the uterus was trimmed free of cervix and oviduct and opened along the mesometrial border. Several sections (~0.5 cm) of both intercaruncular and placentomal uterine wall regions from the midportion of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Several sections (1-1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles, Oneonta, NY), frozen in liquid nitrogen vapor, and stored at -80°C. Remaining placentomes were then removed by physical dissection, and the remaining intercaruncular endometrium was dissected from the myometrium. Endometrial samples were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

Nineteen cyclic ewes were ovariectomized and fitted with intrauterine (i.u.) catheters on Day 5 (Day 0=estrus) and received daily injections (i.m.) of either 50 mg progesterone (P) or P and 75 mg ZK 136.317, a PR antagonist, from Days 5 to 16 and daily i.u. injections of either control (CX) proteins or recombinant oIFN τ (2×10^7 antiviral units/day) from Days 11 to 16 (n=4-5 ewes/treatment). All ewes were hysterectomized on Day 17. At hysterectomy, several sections (~0.5 cm) from the

midportion of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C prior to RNA extraction.

Analysis of RNA

Total cellular RNA was isolated from intercaruncular or placentomal endometria (study 1) and intercaruncular endometrium from treated ewes (study 2) using Trizol (Gibco-BRL, Bethesda, MD) according to manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and the integrity of RNA was examined following gel electrophoresis in a denaturing 1% agarose gel.

Steady-state levels of enJSRV RNAs were assessed by slot blot hybridization as described previously [77]. Denatured total endometrial RNA (10µg) from each ewe was analyzed using a radiolabeled antisense ovine cRNA probe generated from a partial ovine endometrial enJSRV cDNA (DD54) that was cloned by differential display (DD)-PCR as previously described [133]. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S rRNA and cRNA (pT718S; Ambion, Austin, TX). Following washing, nonspecific hybridization was removed by RNase A digestion [77]. The radioactivity associated with each slot was quantified by electronic autoradiography using an Alpha Innotech imaging system and expressed as relative units (RU).

The enJSRV RNA was localized in uterine tissue sections (5µm) from treated animals (study 2) by in situ hybridization analysis as described previously [77]. Deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized ovine endometrial enJSRV cDNA (DD54) or Hyal-2 cDNA [134] by in vitro transcription with [α -³⁵S]UTP. After hybridization, washing, and RNase A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY), stored at 4°C for 1 week, and developed in Kodak D-19 developer. Slides were then counterstained with Harris modified hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated through a graded series of alcohol to xylene, and protected with a coverslip.

Analysis of Protein

enJSRV protein was localized in frozen uterine tissue sections from study 1 by immunofluorescence using methods similar to those described previously [76]. Briefly, uterine tissues embedded in OCT compound from Study 1 were sectioned with a cryostat (8 µm) and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Frozen sections were fixed in -20°C methanol for 10 min, permeabilized with 0.3% Tween 20 in 0.02 M PBS, and then blocked in antibody dilution buffer (2 parts 0.02 M PBS, 1.0% bovine serum albumin, 0.3% Tween 20 [pH 8.0], 1 part glycerol containing 5% normal goat serum for 1 h at room temperature. Sections were rinsed in PBS and incubated overnight at 4°C with the primary antibody against the JSRV envelope protein as described previously [76]. The antibody generated against the JSRV envelope (no. 1721) was produced as described previously [135]. Substitution of primary antibody

with normal rabbit serum (Sigma-Aldrich, St. Louis, MO) at the same concentration was used as the negative control. Following three rinses in PBS for 10 min each, sections were incubated with the fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) (Zymed, San Francisco, CA) for 1 h at room temperature and again washed in PBS three times for 10 min each. Coverslips were placed over a layer of Prolong antifade mounting reagent (Molecular Probes, Eugene, OR).

Statistical Analyses

Data from slot blot hybridization analyses were subjected to least-squares analysis of variance (LS-ANOVA) by the general linear models (GLM) procedures of Statistical Analysis System version 8.1 for Windows (SAS Institute, Cary, NC). Slot blot hybridization data for enJSRV RNAs (RU) were corrected for differences in sample loading by using the 18S rRNA data as a covariate in LS-ANOVA. Data from Study 1 were analyzed for effects of day within the respective tissue type (intercaruncular or placentome). Within tissue type, LS regression analyses were used to determine effects of day on enJSRV RNA levels. Data from Study 2 were analyzed for effects of treatment and their interaction on enJSRV RNA levels. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error [77]. Data are presented as LS mean RUs with standard errors (SE).

Results

Expression of enJSRV RNAs and Protein in the Uterus of Pregnant Ewes

In order to determine the effect of day of pregnancy on enJSRV expression in ovine placentomal and intercaruncular endometrium, steady-state levels of enJSRV

RNAs were determined using slot blot hybridization analysis using the DD54 partial enJSRV envelope cDNA probe [77]. As previously reported, Northern blot analyses of ovine endometrial total RNA with antisense DD54 cRNA detected two RNA species, 7.5 and 2.4 kb in size [58]. If similar to exogenous JSRV, the 7.5 –kb RNA transcript would represent the full-length enJSRV genome, whereas the 2.4-kb RNA transcript corresponds to the correctly spliced *env* mRNA [7].

As illustrated in Figure 4.1, steady-state levels of enJSRV RNAs in both the placentomal and intercaruncular endometrium were affected ($P<0.0001$; $P<0.0017$) by day of pregnancy. Ovine placentomal samples showed an increase in enJSRV RNAs expression as pregnancy progressed from Days 40 to 120. Interestingly, enJSRV RNAs expression in intercaruncular samples decreased from Day 25 to Day 40 of pregnancy and then increased markedly at Day 45. In later pregnancy (Days 60 to 120) enJSRVs RNAs expression in the intercaruncular endometrium remained consistent and was lower than than expressed by the placentomes.

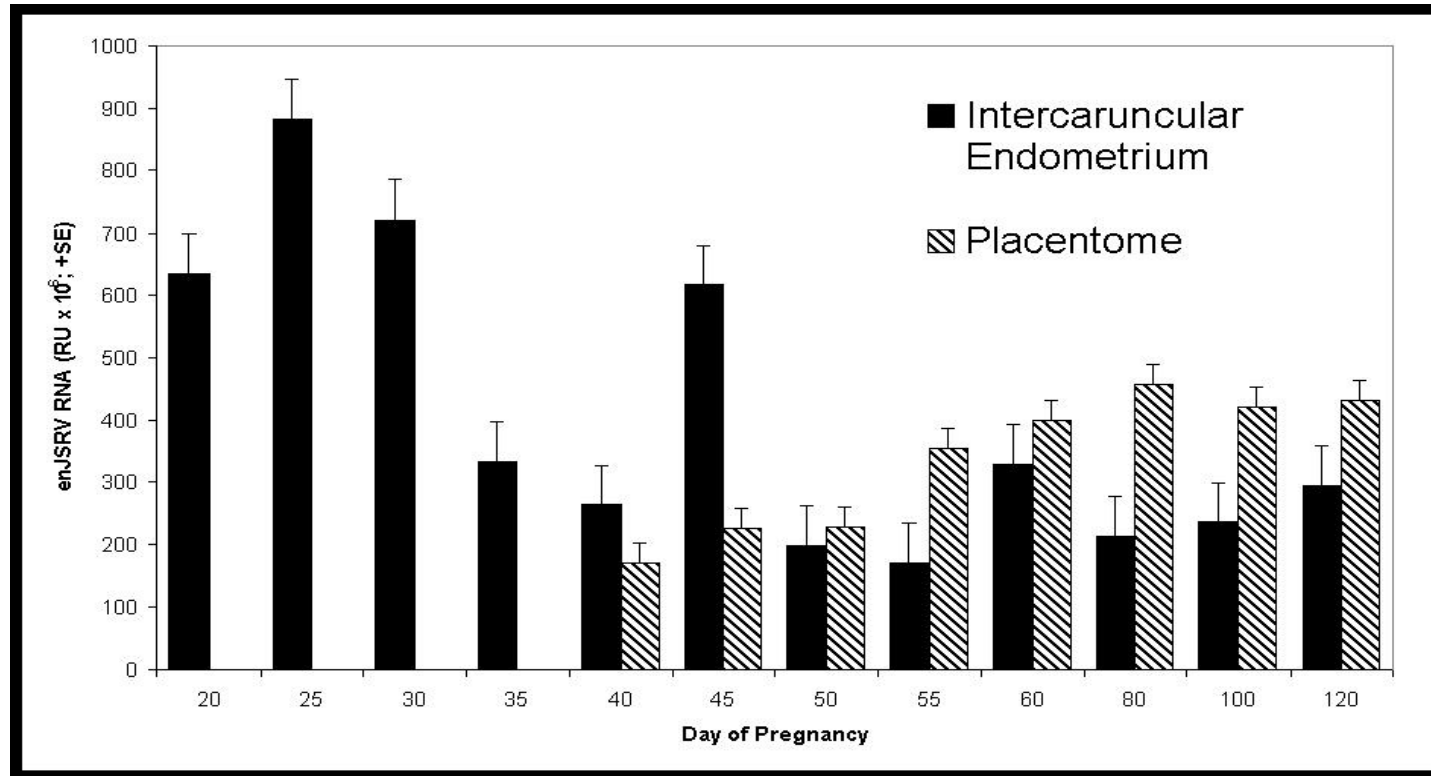


Figure 4.1. Effects of pregnancy on enJSRVs RNA expression in the intercaruncular endometrium and placentomes. Slot blot hybridization analysis of enJSRV RNAs in intercaruncular endometrium and placentomes from pregnant ewes. Steady-state levels of enJSRV mRNAs are presented as LSM relative units (RU) with SE.

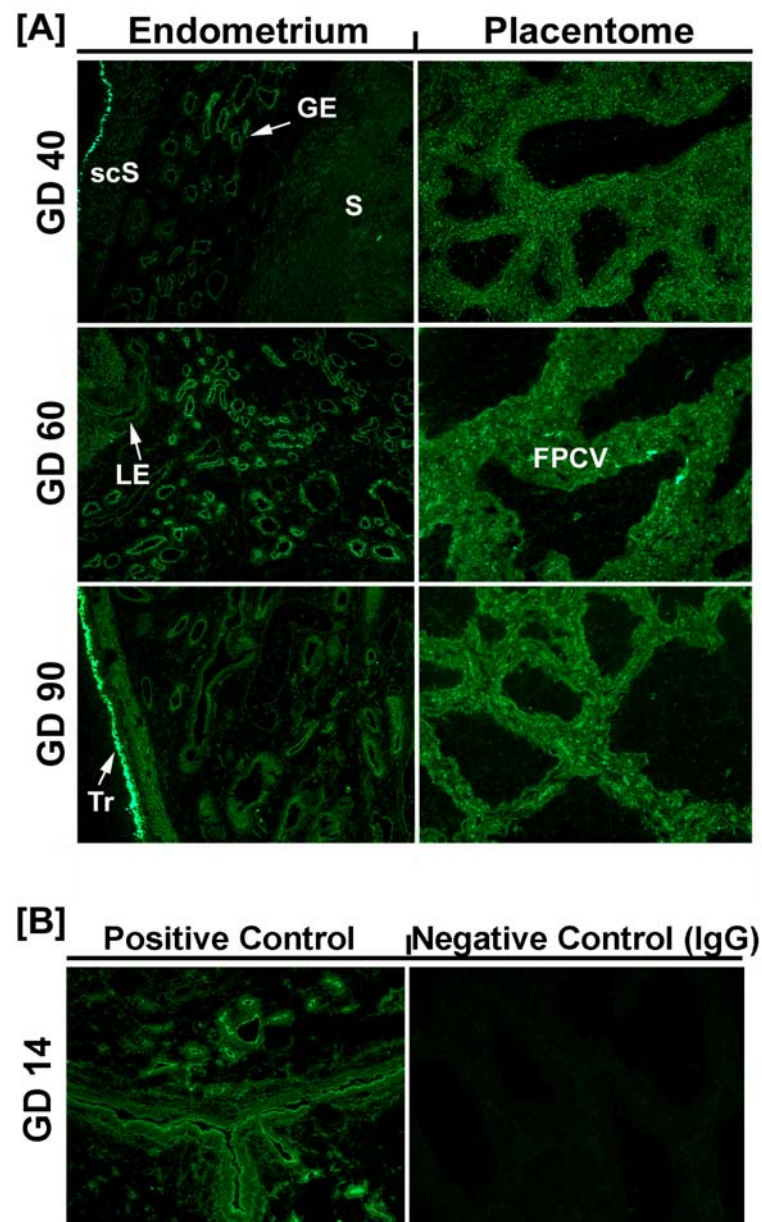


Figure 4.2. Immunoreactive enJSRV envelope like protein expression in the ovine endometrium and placentomes. [A] Immunofluorescence staining of frozen ovine uterine sections from gestational days (GD) 40, 60, and 90 was conducted using to detect the presence of immunoreactive enJSRVs envelope like protein. [B] Uterine cross sections from GD 14 were included to serve as positive control (treated with Env antibody) or negative control (treated with IgG) as described in Materials and Methods. Legend: LE, luminal epithelium; GE, glandular epithelium.; scS, stratum compactum stroma; S, stroma; Tr, trophectoderm; FPCV, fetal placental chorionic villi.

Immunofluorescence analyses of uterine sections from pregnant ewes for JSRV envelope protein expression are presented in Figure 4.2. As observed for enJSRV RNA expression [76], immunoreactive JSRV envelope proteins were most abundant at the maternal/fetal interface. Expression was also evident in the uterine glands as well as the binucleate cells of the conceptus trophoctoderm. Thus the ovine uterine cell types expressing enJSRV RNAs also produced the envelope proteins.

Progesterone Regulation of JSRV RNAs Expression in the Endometrium

Previous research demonstrated that expression of enJSRV genes in the endometrium is highly correlated with circulating levels of progesterone in the peripheral blood and the presence of PR in uterine epithelia [76]. The regulatory effects of progesterone and oIFN τ on the in vivo steady state levels of enJSRV RNAs in the ovine endometrium are depicted in Figure 4.3. Treatment with oIFN τ did not affect ($P>0.10$) endometrial enJSRV RNA levels (P-CX vs P-IFN or P+ZK-CX vs P+ZK-IFN). In contrast, endometrial enJSRV RNA levels were 2-fold higher in P compared to P+ZK ewes, regardless of i.u. protein treatment (P vs P+ZK, $P<0.04$).

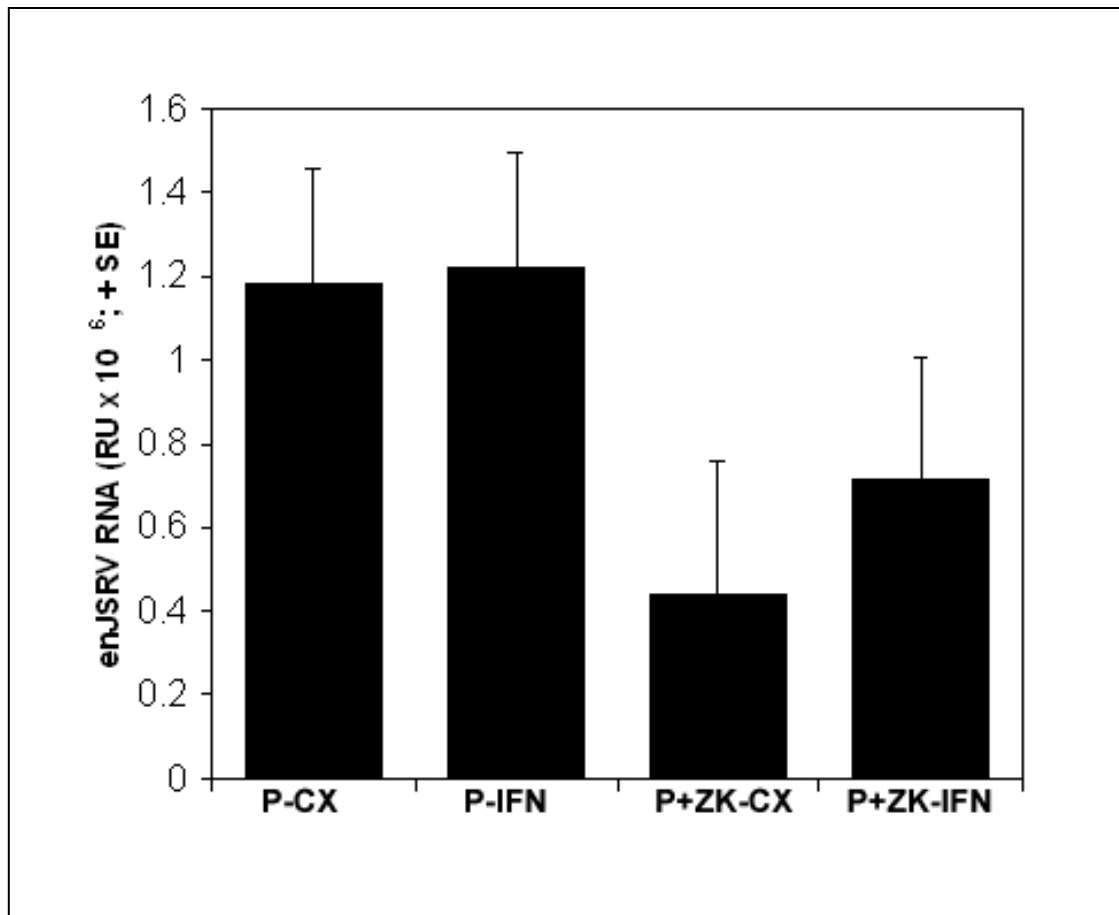


Figure 4.3. Effects of treatment on enJSRVs RNA expression in the endometrium. Slot blot hybridization analysis of enJSRV RNAs in endometrium from treated ewes. Steady-state levels of enJSRV RNAs are presented as LSM relative units (RU) with SE.

In situ hybridization analyses revealed that enJSRV RNA expression was lower in endometrial LE and GE of P+ZK compared to P ewes (Figure 4.4). These in vivo results confirm those from in vitro experiments conducted by Palmarini et al., [136], indicating that P, acting through PR, increases expression of enJSRVs in LE and GE in a temporal manner coincident with the beginning of conceptus elongation and implantation.

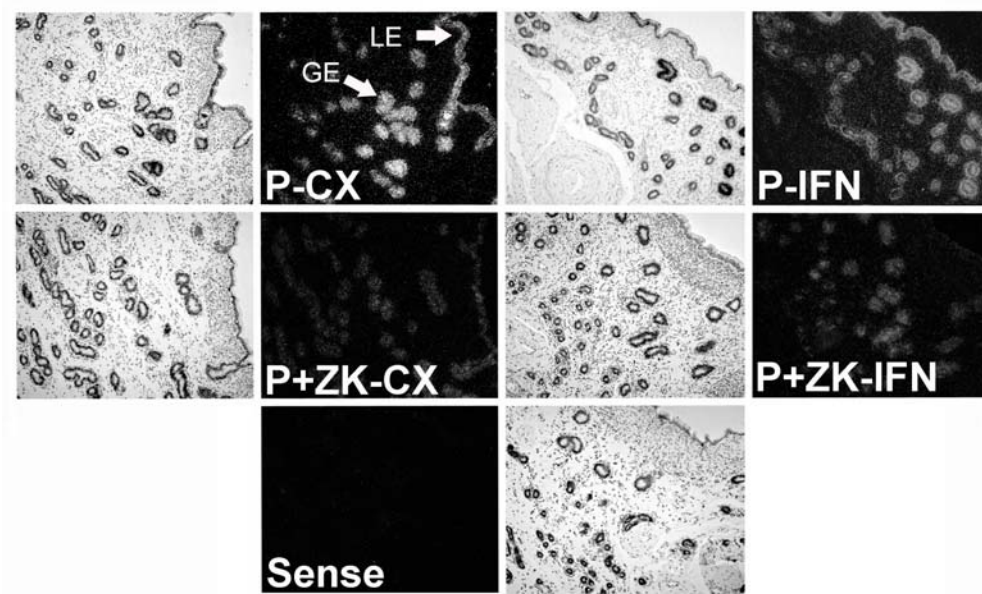


Figure 4.4. *In situ* hybridization analysis of enJSRVs RNA expression in uteri of treated ewes. Cross-sections of the uterine wall were hybridized with radiolabeled antisense or sense ovine enJSRV cRNA probes. Protected transcripts visualized by liquid emulsion autoradiography for one week and imaged under brightfield or darkfield illumination. Legend: LE, luminal epithelium; GE, glandular epithelium.

Discussion

The present studies assessed the influence of progesterone, interferon tau, and stage of pregnancy on expression of enJSRVs, as an effort to understand the significance of enJSRVs in reproductive biology. Available results suggest that enJSRVs are involved in ovine reproduction. These studies provide temporal and spatial evidence for progesterone regulation of the enJSRVs envelope in the ovine uterus.

Previous research has demonstrated that changes in steady-state levels of enJSRV RNAs closely parallel changes in concentration of progesterone in the

peripheral circulation as well as expression of the progesterone receptor in endometrial epithelia [76]. Specifically, expression of enJSRV RNAs in the endometrial epithelia increased 12-fold between Days 1 and 13 of the estrous cycle and pregnancy [76]. Also previously shown in pregnant ewes was high endometrial enJSRV RNA expression on Day 11, increasing to maximum value on Day 13, and then decreasing to Day 19 [76, 80, 81]. The increase in epithelial enJSRVs expression occurs during the period when the blastocysts hatch from the zona pellucida on Day 9, transition from spherical to tubular conceptuses by Day 12, and then undergoes rapid elongation beginning on Day 12 to filamentous conceptuses that occupy the entire uterine horn by Day 16 [79]. These development changes in conceptus development involve rearrangement and proliferation of the mononuclear trophoctoderm cells as well as production of the pregnancy recognition signal, interferon tau (IFN τ) [96].

Investigation of the effects of progesterone and interferon tau on enJSRV expression in vivo indicated that enJSRVs expression was directly regulated by progesterone and progesterone receptor in the ovine endometrial epithelia. In situ hybridization analyses of uteri revealed that enJSRV RNA expression levels were lower in endometrial LE and GE of ewe that received the progesterone receptor antagonist (ZK) as compared to ewes that received progesterone only. No effects of IFN τ on enJSRVs RNA expression were detected in ewes receiving either P or P+ZK. These in vivo results confirm those from in vitro experiments indicating that progesterone, acting through PR, increases expression of enJSRVs in endometrial LE and GE in a temporal manner coincident with the beginning of conceptus elongation and implantation.

While previous research has shown that expression of enJSRVs in the ovine uterus coincides with blastocyst maturation and the peri-implantation period it was also important to evaluate enJSRVs expression throughout later gestation. Intercaruncular endometrial tissues collected during the later stages of gestation revealed that enJSRV expression was high at Days 25, 30, and 45 prior to decreasing throughout the remainder of gestation. *In situ* hybridization revealed that the localization of enJSRV expression in LE and GE also changed as gestation progressed. As expected, the loss of the LE during synepitheliochorial placentation at Day 16 resulted in a shift of enJSRV expression levels from LE to GE. Also evidenced by immunofluorescence was enJSRV expression in the developing trophoctoderm suggesting that enJSRVs may be necessary for successful formation of syncytia.

Further evidence for a role of enJSRVs in gestation was the relatively consistent levels of enJSRV expression in placentomes throughout the later stages of gestation. The placentome is a structure formed during placentation in ruminants from the interdigitation and branching morphogenesis of a placental cotyledon, containing binucleate cells, with a maternal endometrial caruncle [7]. *In situ* hybridization of placentomal samples revealed enJSRV expression in both the fetal cotyledon and the maternal caruncle [7]. High levels of enJSRV expression within the binucleate cells were also demonstrated [96]. Binucleate cells are formed from mononuclear cells of the trophoblast, which duplicate their DNA without dividing [7, 76]. Interestingly, through the use of immunofluorescence, we detected both enJSRV RNAs and envelope protein in the sheep placental binucleate cells, similar to results obtained in early gestation [79].

Only the binucleate cells display invasive properties in placentae of ruminants and they are abundantly present in the placentome. In addition, the binucleate cells alone synthesize and secrete placental lactogen, a key hormone in pregnancy that stimulates endometrial gland morphogenesis and differentiated function for fetal nutrition [6, 113]. The results of this study reveal that expression of enJSRVs is modulated by progesterone, but not IFN τ in vivo. The relative expression of enJSRVs, their corresponding temporal and spatial localization at the maternal/fetal interface during placentation, and their regulation by progesterone suggests that they play key roles in the reproductive physiology of sheep during placentation. These data add further support to the validity of the ewe as a model to understand the biological significance of endogenous retrovirus with respect to regulation of conceptus development, implantation, and pregnancy recognition signaling.

CHAPTER V

ENDOGENOUS RETROVIRUSES REGULATE PERI-IMPLANTATION PLACENTAL GROWTH AND DIFFERENTIATION

Introduction

The sheep genome contains approximately 20 copies of ERVs highly related to the exogenous and pathogenic Jaagsiekte sheep retrovirus (JSRV; hence the name enJSRVs) [113]. enJSRVs RNA and proteins are abundantly expressed in the epithelia of the female genital tract [76]. In the placenta, enJSRVs are expressed in the mononuclear trophoctoderm cells, but are most abundant in the trophoblast giant binucleate cells (BNC) and multinucleated syncytial plaques of the placentomes . The temporal expression of enJSRVs in the trophoblast is coincident with key events in the development of the sheep conceptus (embryo/fetus and associated extraembryonic membranes). enJSRVs mRNAs are first detected at day 12 [7], when the blastocyst begins the process of elongation, involving intense proliferation and outgrowth of mononuclear trophoctoderm cells producing interferon tau (IFNT), the antiluteolytic signal for pregnancy recognition in ruminants .

In sheep, BNC differentiate from mononuclear trophoctoderm cells between days 14 and 16, migrate and then fuse with the uterine luminal epithelium as well as each other to form syncytial plaques that ultimately form the cotyledonary portions of the placenta . The BNC derive from the mononuclear trophoctoderm cells by a poorly characterized mechanism presumably involving mitotic polyploidy, whereas the syncytial plaques are

though to develop by cell-cell fusion [85]. Hyaluronidase 2 (HYAL2) is a glycosylphosphatidylinositol-anchored cell-surface protein that can serve as a cellular receptor for the exogenous JSRV Env as well as for retroviral vectors pseudotyped by enJSRVs Env . By RT-PCR analyses, *HYAL2* mRNA is found in the conceptus only after day 14, which is associated with the onset of trophoblast giant BNC differentiation. Throughout pregnancy, *HYAL2* mRNA can be detected in the BNC and multinucleated syncytia of the sheep placentomes, but not in the mononuclear trophoblast or uterine luminal epithelium.

Of great interest for comparative physiology is that enJSRVs expression in the developing ovine trophoblast and placenta is strikingly similar to that observed for syncytin 1 and 2, products of HERV-W *env* in humans and primates and possibly of two related *env* genes (*syncytin-A* and *syncytin-B*) in mice . Syncytins encode highly fusogenic retroviral envelope proteins that are expressed in the syncytiotrophoblast layer generated by mononuclear cytotrophoblast cell fusion at the maternal-fetal interface. Syncytins are fusogenic when expressed in vitro, thereby advancing the hypothesis that they are involved in placental morphogenesis . Thus, circumstantial evidence gleaned from studies of sheep, primates and rodents supports the concept that independently acquired ERVs have been positively selected for a convergent physiological role in placental morphogenesis [27].

Studies to determine the biological role of genes expressed in the conceptus of domestic animals have been hampered by the inability to perform loss-of-function studies. Although gene targeting can now be performed in domestic animals, the

technique is extremely inefficient and not practical at present for research studies with single genes and particularly with the multiple enJSRVs loci. Recently, Luu et al. [137] demonstrated the usefulness of morpholino antisense oligonucleotides in the mouse uterus in vivo to knockdown expression of calbindin-9K protein in endometrial epithelia of pregnant mice which prevented implantation. This morpholino approach to loss-of-function studies has been adapted by our laboratory for use in sheep.

Morpholino oligos are short chains of morpholino subunits comprised of a nucleic acid base, a morpholine ring, and a non-ionic phosphorodiamidate intersubunit linkage (Gene Tools, Philomath, OR; www.gene-tools.com). Morpholinos act via a steric block mechanism (RNase H-independent) that inhibits the translation initiation complex (by targeting the 5' untranslated region or UTR through the first 25 bases of coding sequence) [138]. Their high mRNA binding affinity and exquisite specificity yields reliable and predictable results. There is no other gene knockdown reagent (including siRNA, PNA, mPNA, S-DNA, and LNA) that combines the properties of stability, nuclease-resistance, efficacy, long-term activity, water-solubility, low toxicity, and exquisite specificity. The traditional approach to the use of morpholinos has been to deliver them into cells by microinjection. Effective in vivo delivery of morpholinos has been enabled using endocytotic delivery with Endo-Porter. Endo-Porter is a novel peptide specifically designed to deliver substances (morpholinos, peptides, proteins, antibodies) into the cytosol of cells by an endocytosis-mediated process that avoids damaging the plasma membrane and works with serum (Gene Tools).

In these studies, we tested the hypothesis that enJSRVs Env have a biological

role in peri-implantation ovine conceptus development and placental morphogenesis using an *in vivo* morpholino loss-of-function approach to block enJSRVs Env production that has been successfully utilized in mice to examine the function of implantation-related genes *in utero* [137, 139].

Materials and Methods

Morpholino Design

Morpholino oligonucleotides were designed and synthesized by Gene Tools, LLC (Philomath, Oregon, USA). The MAO-env had the sequence GCTTC GGCAT CCTGT GGAAA AACAC and targeted to the enJSRVs *env* RNA overlapping the splice donor and acceptor region (see Fig. 5A). The MAO-5mis control morpholino had the sequence GGTTC GCCAT CCTCT GCAAA AAGAC (underlined differences with MAO-env). The MAO-std had the sequence CCTCT TACCT CAGTT ACAAT TTATA and targeted to a splice site mutant of Homo sapiens hemoglobin beta (*HBB*) gene (AY605051). All morpholinos were synthesized with a 5' rhodamine modification for convenient detection.

In vitro Transfection Studies

pSV-En2EnvFlag expresses, under the control of the SV40 promoter, the Env of enJS5F16 tagged with a FLAG epitope at the carboxyterminal,. pCMV2en56A1 expresses the full length enJS56A1 locus . Human 293T cells were transfected with pSV-En2EnvFlag or cotransfected with pSV-En2EnvFlag and pCMV2en56A1 using lipofectamine (Invitrogen). After 3 h, cells were washed with PBS and incubated with Endo-Porter Aqueous delivery reagent (GeneTools) (8 μ l/ml of medium) and 20-80 μ M

of MAO-env, MAO-5mis or MAO-std. After 48 h, transfected cells were lysed, and cell lysates were analyzed for the presence of enJSRV Env or Gag by immunoprecipitation and/or western blotting employing an anti-Flag antibody (Sigma-Aldrich) and an anti-JSRV p23 (Matrix) as previously described .

In vivo Studies

All animal experiments were approved by the Institutional Animal Care and Use Committee of Texas A&M University. Suffolk cross-bred ewes were mated at estrus (day 0) and on day 1 to rams of proven fertility. On day 8 post-mating, the ewes were subjected to a mid-ventral laparotomy. The base of the uterine horn ipsilateral to the corpus luteum was double ligated using non-absorbable umbilical tape to prevent migration and growth of the conceptus through the uterine body into the contralateral uterine horn. This surgical procedure does not affect conceptus implantation or fetal development in sheep. MAO-std, MAO-5mis, and MAO-env (100 nmol) were complexed with Gene Tools Endo-Porter delivery reagent (50 μ l) and diluted to 1 ml final volume with OPTI-MEM (Invitrogen). The complex was then introduced into the lumen of the uterus (n=5-6 ewes/morpholino) via the uterotubal junction using a 1 ml syringe fitted with a 20-gauge catheter. After discharging the morpholinos into the uterine lumen, the catheter was withdrawn, and the uterine horn gently massaged to distribute the morpholinos throughout the uterine lumen. The outside of the uterus was rinsed with sterile 5% glycerol in saline to prevent formation of adhesions and placed back in the body cavity.

For Study One, the morpholino-injected ewes were hysterectomized on day 16. The

uterine horn injected with the morpholinos was flushed with 10 ml sterile phosphate buffered saline (PBS). If present, the morphology of the conceptus was recorded (spherical, tubular, or elongated). The conceptus was immediately removed from the uterine flush with a pipette, and the volume of the flush recorded. Photomicrographs of the conceptus were obtained using an inverted microscope fitted with a digital camera. Portions of the conceptus were then placed in Optimal Cutting Temperature (OCT) compound (Miles, Oneonta, NY), frozen in liquid nitrogen, and stored at -80°C. Another portion of the conceptus was fixed in freshly prepared 4% paraformaldehyde in PBS and embedded in paraffin wax. The uterine flush was clarified by centrifugation (5000 x g for 15 min at 4°C), aliquoted and stored at -80°C. The amount of IFNT in the uterine flush was quantified by semi-quantitative Western blot analysis.

For Study Two, the morpholino-injected ewes were hysterectomized on day 20. The uterine horn injected with the morpholinos was not flushed, but rather opened along the mesometrial border to expose the conceptus. Portions of the conceptus (if present) and sections of the uterine horn containing the conceptus were frozen in OCT compound or fixed in 4% paraformaldehyde for histological analysis.

Histology, Immunohistochemistry and Immunofluorescence

Effective delivery of the rhodamine-labeled morpholinos was analyzed by fluorescence microscopy. Cryosections of the uteri and conceptuses were prepared, and a DAPI-containing mounting medium used to visualize nuclei. The enJSRV Env and Gag proteins were evaluated in frozen conceptus tissue sections by immunofluorescence staining using a rabbit antiserum towards the JSRV Env or Gag as described previously ;

rabbit IgG was used in place of the primary antibody as a negative control. Immunoreactive PL and PAG proteins were assessed in the conceptus using standard immunohistochemical procedures with antibodies to ovine CSH1 (rabbit polyclonal kindly provided by Dr. Russ V. Anthony, Colorado State University) and ovine PAG (rabbit anti-recombinant PAG-3 polyclonal kindly provided by Dr. Jonathan A. Green, University of Missouri-Columbia). Rabbit IgG was used in place of the relevant primary antibody as a negative control. The number of BNC was quantified by determining the number of CSH1- and PAG-immunostained BNC relative to mononuclear trophoblast cells in at least five non-sequential sections of each conceptus from each ewe. The nuclei were visualized after applying hematoxylin counterstain.

In vitro Ovine Mononuclear Trophectoderm Cell Culture and Proliferation Assay

Conceptuses from Day 15 pregnant ewes were recovered by sterile flush. The inner cell mass removed by dissection, and trophectoderm cells were isolated and cultured using methods described previously [140]. Rhodamine-labeled MAO-std (100 nmol) was complexed with Endo-Porter aqueous delivery reagent (6 μ l/ml medium) and added to cells in culture. Fluorescence microscopy was used to visualize the labeled MAO in cells 24 hours after treatment using a Zeiss Stallion Double Detector Imaging system (Carl Zeiss Microimaging, Thornwood, NY).

For immunofluorescence analyses, cells were grown on 2 well Lab-Tek Coverglass Chambered slides (Nalge Nunc, Rochester, NY) and mock-treated (no morpholino) or treated with MAO-std, MAO-5mis or MAO-env (100 nmol) complexed with Endo-Porter aqueous delivery reagent for 48 h. Immunoreactive enJSRVs Env and

Gag proteins were analyzed by immunofluorescence staining using a rabbit antiserum towards the JSRV Env or Gag as described previously ; rabbit IgG was used in place of the primary antibody as a negative control. Cells were sequentially imaged using a Cy3 filter set followed by differential interference contrast optics using either a Planachromat 10X/0.45 or a C-Apochromat 63X/1.2 W. Corr objective lens. The experiment was independently repeated three times.

To determine effects of morpholinos on trophectoderm cell proliferation, cells were grown in 6-well culture dishes until 30% confluency and mock-treated (no morpholino) or treated with MAO-std, MAO-5mis or MAO-env (100 nmol complexed to Endo-Porter Aqueous Delivery Reagent) in triplicate for 48 h. A colorimetric assay using Janus green dye was used to assess cell numbers. The experiment was independently repeated three times.

IFN τ Determination by Western Slot Blot Analysis

Uterine flushings from each Day 16 ewe were clarified by centrifugation (3000 x g for 30 min at 4°C), aliquoted and stored at -80°C until analysis. Uterine flushes were thawed on ice. An equal volume of each flush was affixed to a nylon membrane using a slot blot apparatus. After drying, the membrane was analyzed by standard Western blot analysis using methods described previously[138, 141] and rabbit antiserum to recombinant ovine IFN τ [142]. Immunoreactive IFN τ protein was detected using enhanced chemiluminescence and quantified using a Typhoon 8600 imager (GE Healthcare). Data are presented as relative units (RU).

Statistics

Pregnancy rate data were analyzed by Chi square. All quantitative data were subjected to least squares analysis of variance (LS-ANOVA) using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Statistical models accounted for sources of variation including main effects of morpholino type and, where appropriate, histological section (BNC quantification) or replicate (proliferation assays). Data are presented as least squares means with standard error of the mean (s.e.m.).

Results

Effects of morpholinos on enJSRV Env expression in vitro

A morpholino antisense oligonucleotide (MAO) was designed to specifically inhibit expression of enJSRVs *env* mRNAs (MAO-env) (Figure. 5.1).

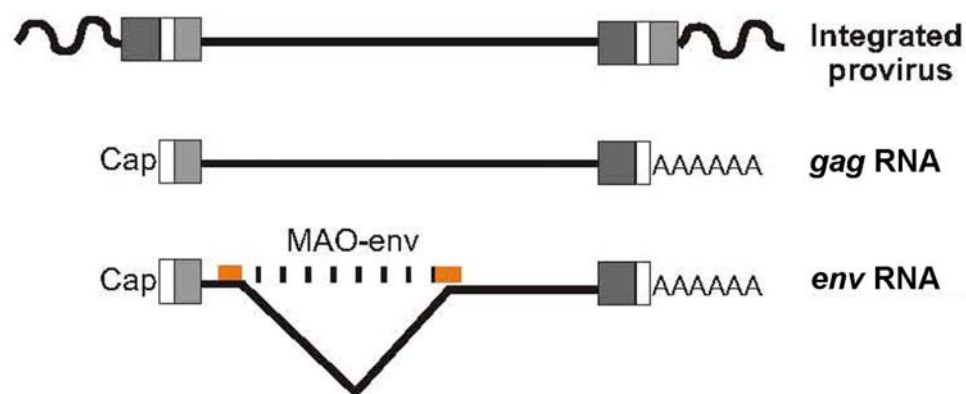


Figure 5.1. Morpholino design. MAO-env was designed to inhibit the splicing and translation of the enJSRVs *env* mRNA, but does not block expression of full length mRNA (which expresses the viral Gag).

Morpholino antisense oligonucleotides inhibit RNA splicing and/or translation via a steric block mechanism that is RNase H-independent [138]. Morpholinos are effective only when designed to complement the nucleotide region around the start codon and/or possible splicing sites of a given gene mRNA. The nucleotide sequence around the splice acceptor and start codon of the exogenous JSRV *env* and the known enJSRV loci are highly conserved, indicating that one common MAO should inhibit splicing and translation of most enJSRV proviral loci expressing an intact *env* gene. To examine morpholino effectiveness, we conducted a series of in vitro studies in transiently transfected 293T cells. 293T cells were mock transfected or transfected with pSV-En2EnvFlag, an SV40 driven expression plasmid for enJS5F16 *env* cDNA tagged with a FLAG epitope at the C-terminus (Figure 5.2A). Cells were then treated with either, MAO-*env*, MAO-5mis, or MAO-std (Figure 5.2A). All morpholinos were complexed with the Endo-Porter delivery reagent and used at a final concentration of 80 μ M. After 48 h, enJS5F156 Env expression was determined by immunoprecipitation and Western blot analysis. To further examine effectiveness 293T cells were mock-transfected or transfected with pSV-En2EnvFlag as above (Figure 5.2B). Cells were then treated with either Endo-Porter alone, MAO-std, MAO-5mis, or MAO-*env* (Figure 3.2B). All morpholinos (20, 40 and 80 μ M) were complexed with the Endo-Porter delivery reagent and after 24 h, enJS5F16 Env expression was determined by immunoprecipitation and Western blot analysis (Figure 5.2B). Summarily, MAO-*env* effectively inhibited expression of the enJS5F16 (one of the cloned enJSRV loci) *env* (Figure 3.2A) in a dose-dependent fashion (Figure 5.2B). A 5-mismatch (MAO-5mis) and a standard

(MAO-std) control morpholino had no effect on expression of the enJS5F16 *env* (Figure 5.2).

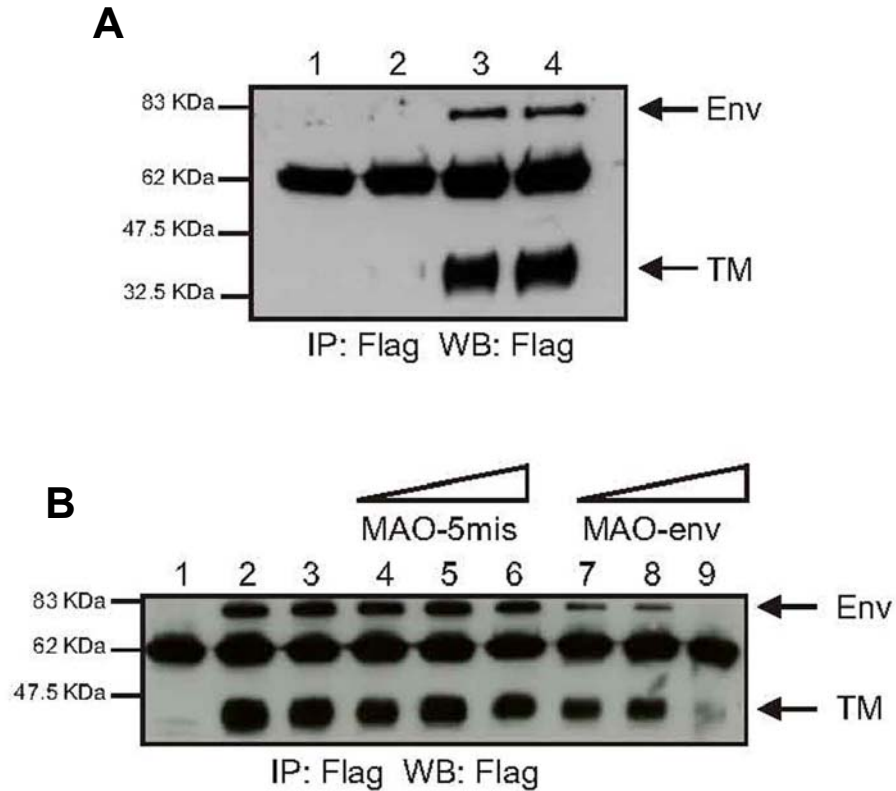


Figure 5.2. Effects of MAO on enJSRV Env *in vitro*. (A) 293T cells were mock transfected or (lane 1) or transfected with pSV-En2EnvFlag, an SV40 driven expression plasmid for enJS5F16 *env* cDNA tagged with a FLAG epitope at the C-terminus (lanes 2-4). Cells were then treated with MAO-env (lane 2), MAO-5mis as a control (lane 3) or MAO-std as a control (lane 4). All morpholinos were complexed with the Endo-Porter delivery reagent and used at a final concentration of 80 μ M. After 48 h, enJS5F156 Env expression was determined by immunoprecipitation and Western blot analysis. Note that the full length retroviral Env is processed into a surface domain and a transmembrane domain (TM). (B) 293T cells were mock-transfected or transfected with pSV-En2EnvFlag as above (mock; Lane 1). Cells were then treated with Endo-Porter alone (Lane 2), MAO-std as a control (Lane 3), MAO-5mis as a control (Lanes 4-6; 20, 40 and 80 μ M), or MAO-env (Lanes 7-9; 20, 40 and 80 μ M). All morpholinos were complexed with the Endo-Porter delivery reagent. After 24 h, enJS5F16 Env expression was determined by immunoprecipitation and Western blot analysis as in A.

Effects of MAO on enJSRV Gag expression in vitro

To assess MAO specificity 293T cells were mock transfected or co-transfected with pSV-En2EnvFlag and pCMV2-enJS56A1 expressing the full length enJS56A1 clone prior to treatment with either Endo-Porter alone, MAO-5mis, or MAO-Env (Figure 5.3). All morpholinos were complexed with the Endo-Porter delivery reagent and used at a final concentration of 80 μ M. After 48 h, enJSRV Env expression was determined by immunoprecipitation and Western blot analysis and Gag expression by Western blot analysis (Figure 5.3). The results demonstrate that the, MAO-env and the control morpholinos did not affect expression of enJSRV *gag* (the polyprotein forming the retroviral capsid) in transfected 293T cells (Figure 5.3), since the retroviral Gag is produced from a full-length genomic mRNA, whereas Env is only produced from correctly spliced mRNA (Figure 5.1). Thus, MAO-env specifically inhibits enJSRVs *env* mRNA translation.

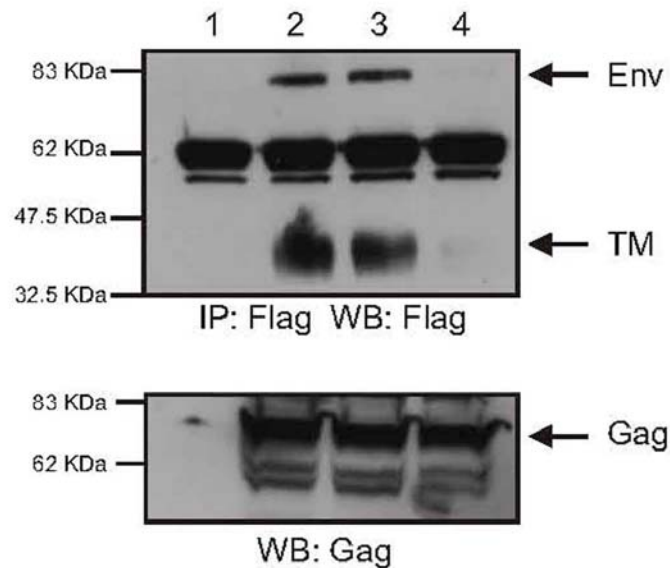


Figure 5.3. Effects of MAO on enJSRV Gag expression *in vitro*. 293T cells were mock transfected (lane 1) or co-transfected with pSV-En2EnvFlag and pCMV2-enJS56A1 expressing the full length enJS56A1 clone (Lanes 2-4). Cells were then treated Endo-Porter alone (Lanes 1 and 2), MAO-5mis as a control (Lane 3), or MAO-Env (Lane 4). All morpholinos were complexed with the Endo-Porter delivery reagent and used at a final concentration of 80 μ M. After 48 h, enJSRV Env expression (upper panel) was determined by immunoprecipitation and Western blot analysis as in Figure 3.2 and Gag expression by Western blot analysis (lower panel).

To conduct *in vivo* loss-of-function studies, we injected the morpholinos into the lumen of the ovine uterus on day 8 post-mating and determined their effect on conceptus development and pregnancy establishment on either day 16 (Study One) or day 20 (Study Two). In Study One, pregnancy rates were not different ($p>0.10$) among the various treatments (Table 5.1).

Table 5.1. Effect of morpholinos on pregnancy in sheep

Morpholino	Study One: Day 16 Post-Mating			
	Pregnancy rates¹	Conceptus development	IFNτ Levels	BNC (%)
MAO-std	100% (5/5)	Elongated, Filamentous ² ; +++	282 \pm 50	12 \pm 1
MAO-5mis	100% (5/5)	Elongated, Filamentous ² ; +++	232 \pm 50	10 \pm 1
MAO-env	100% (5/5)	Growth-retarded, Filamentous \pm	68 \pm 50	1 \pm 1
	Study Two: Day 20 Post-Mating			
MAO-std	100% (5/5)	Elongated, filamentous ² ; +++	n.d.	n.d.
MAO-5mis	83% (5/6)	Elongated, filamentous ² ; +++	n.d.	n.d.
MAO-env	20% (1/5)	Not present ³	n.d.	n.d.

¹All ewes received intrauterine injections of morpholinos on Day 8 post-mating and were hysterectomized on either Day 16 or Day 20. Data include the percentage of ewes with a conceptus in the uterine lumen at hysterectomy (pregnant/total). ²+++ = normal morphology and development of the conceptuses; \pm = conceptuses were growth retarded, slightly elongated and fragile with no detectable trophoblast giant binucleate cells (BNC). ³The single recovered conceptus was severely growth retarded as compared to conceptuses recovered from MAO-5mis and MAO-std controls as well as non-injected horn.

However, major differences were observed in conceptus development in ewes receiving the MAO-env as compared to MAO-5mis or MAO-std controls. The conceptuses from all ewes injected with the control morpholinos (MAO-5mis and MAO-std) were fully elongated and filamentous, which is typical of day 16 of pregnancy (Figure 5.4A). Although the conceptuses recovered from ewes injected with the MAO-env were elongated, they were fragile and substantially smaller than those from ewes injected with control morpholinos (Figure 5.4A). Histological examination found that conceptuses from ewes injected with either control morpholino contained many mononuclear trophoderm cells (Figure 5.4B). The conceptuses from MAO-env treated ewes had mononuclear trophoderm cells, many of which displayed intracytoplasmic vacuoles (Figure 5.4B).

In order to quantify the effect of MAO-env on mononuclear trophoderm cell growth, we measured the levels of IFNT (which is solely produced by mononuclear trophoderm cells) in uterine flushings. Consistent with retarded growth of the conceptus (Figure 5.4A), the relative amounts of IFNT in the uterine flushings of MAO-env injected ewes (68 ± 10 relative units) were considerably lower ($p < 0.05$) than MAO-5mis (232 ± 50 relative units) and MAO-std (282 ± 50 relative units) injected ewes (Table 5.1).

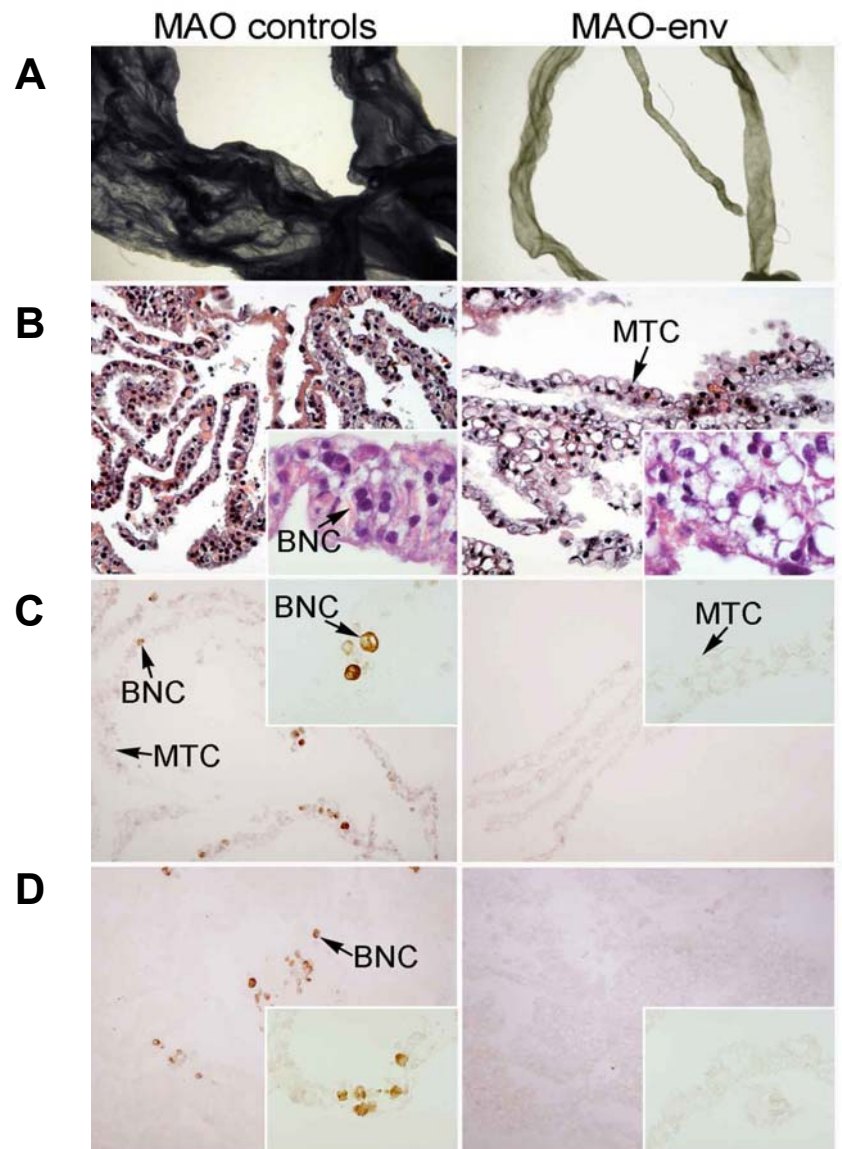


Figure 5.4. Effects of morpholinos on peri-implantation conceptus trophoblast growth and differentiation. MAO-std, MAO-5mis or MAO-env were injected into the uterine lumen on day 8 post-mating and recovered on day 16. **A**, Gross morphology. Morphology of the conceptuses was examined using an inverted microscope (shown at same magnification). **B**, Histology. Portions of the conceptuses were fixed in paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Width of each field of view is equal to 420 μ m with the inset at 85 μ m. MTC, mononuclear trophoblast cell; BNC, trophoblast giant binucleate cell. **C**, Pregnancy-associated glycoproteins (PAGs). **D**, Placental lactogen (PL) in the conceptus. Immunoreactive PAG and PL protein was detected in paraformaldehyde-fixed, paraffin-embedded sections of conceptuses using a rabbit anti-ovine PAG or anti-ovine PL antibody. Width of each field of view is equal to 420 μ m with the inset at 210 μ m. Data are representative of conceptuses from all ewes.

Next, we evaluated conceptuses for the presence of trophoblast giant BNC, which derive from mononuclear trophoctoderm cells. Chorionic somatomammotropin one (CSH1; alias placental lactogen) and pregnancy-associated glycoproteins (PAG) are only expressed by the BNC and are useful markers for these cells [81]. Immunostaining for CSH1 and PAG proteins found $10 \pm 1\%$ or $12 \pm 1\%$ BNC in the conceptuses from control ewes (MAO-5mis and MAO-std, respectively), whereas BNC were very scarce ($1 \pm 1\%$) or not present at all in conceptuses recovered from MAO-env injected ewes (Figure 5.4C, 5.4D). Therefore, in vivo enJSRVs Env knockdown in the conceptus retarded trophoctoderm growth and inhibited differentiation of trophoblast giant BNC.

Next we analyzed whether retarded development of conceptuses of MAO-env treated ewes was associated with a reduction in enJSRVs Env expression (Figure 5.5). As shown in Figure 5.5A, the rhodamine-labeled morpholinos were observed in trophoctoderm cells of the conceptus as well as in luminal epithelium and superficial glandular epithelium of the endometrium. In conceptuses recovered from ewes injected with MAO-5mis and MAO-std controls, abundant enJSRV Env protein was observed at the apical surfaces of the mononuclear trophoctoderm cells as well as the luminal epithelium and glandular epithelium of the endometrium.

As assessed by immunofluorescence analysis, enJSRV Env expression was almost completely diminished in the trophectoderm of day 16 conceptuses recovered from ewes injected with MAO-env and was also substantially decreased in the uterine luminal epithelium (Figure 5.5B). Immunofluorescence analyses are required for evaluation of enJSRVs Env abundance with the rabbit anti-JSRV Env antibody, because it does not recognize the Env protein under Western blot conditions (unpublished results). No differences in enJSRVs Env expression was observed in the epithelia of the middle to deep uterine glandular epithelia, which was expected due to the inability of the morpholinos to be delivered to those cells (data not shown). As expected from in vitro results (Figure 5.1), expression of enJSRVs Gag protein in the conceptus trophectoderm and endometrial epithelia was not affected by the morpholinos (Figure 5.5C). Thus, the observed alterations in conceptus development, trophectoderm growth, and BNC differentiation in MAO-env treated ewes were directly correlated with inhibition of enJSRVs Env by the MAO-env approach.

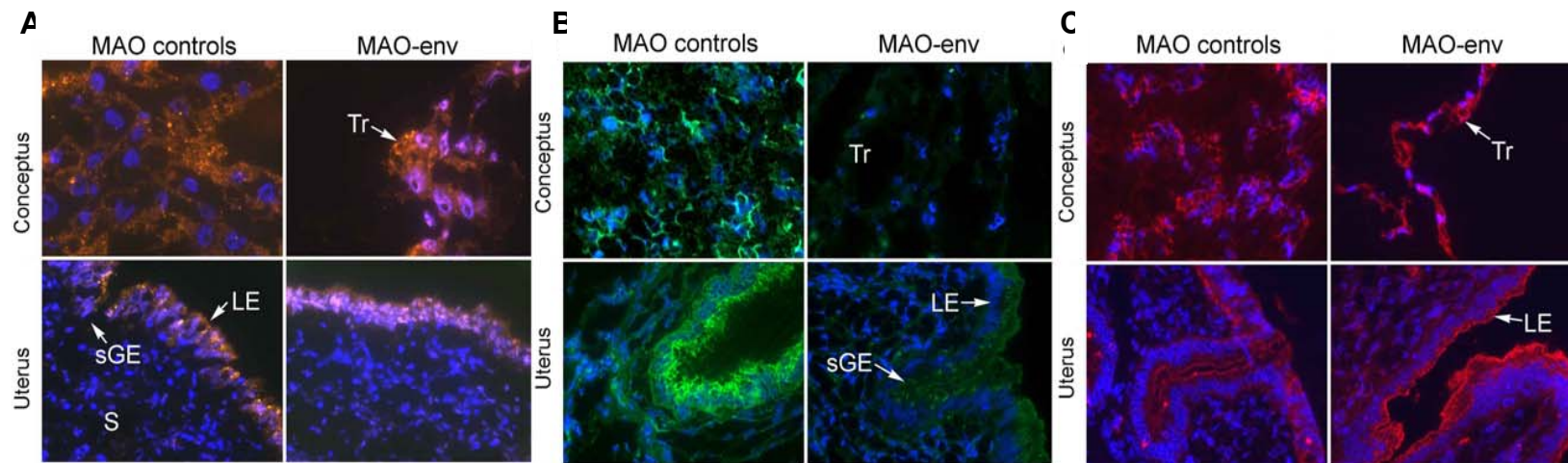


Figure 5.5. *In vivo* delivery and effectiveness of morpholinos. MAO-std, MAO-5mis or MAO-env was injected into the uterine lumen of sheep on Day 8 post-mating. The conceptuses were removed on Day 16. **A**, Fluorescence microscopy analysis of morpholino delivery. Portions of the conceptuses were frozen in OCT and sectioned. Sections were rinsed in phosphate-buffered saline and a coverslip affixed using DAPI-containing mounting medium. Fluorescence microscopy was used to detect the rhodamine-labeled morpholino (orange/red) and DAPI nuclei (blue). **B**, enJSRV Env protein. Conceptuses and uteri were sectioned, and analyzed for enJSRV Env protein by immunofluorescence analysis using a rabbit anti-JSRV surface (SU) Env antibody with a FITC-labeled secondary antibody (63x magnification). **C**, enJSRV Gag protein. Conceptuses and uteri were sectioned, and analyzed for enJSRV Gag protein by immunofluorescence analysis using a rabbit anti-JSRV Gag antibody with a FITC-labeled secondary antibody. Width of each field of view is equal to 140 μ m. Data are representative of conceptuses from all ewes.

In Study Two, we determined whether conceptuses from MAO-env treated ewes could establish pregnancy. Ewes were injected with morpholinos on day 8 post-mating and effects assessed on day 20. Early pregnancy loss occurred in almost all MAO-env injected ewes, but not in control ewes. Pregnancy rates of 100% and 83% were not different ($p>0.10$) in control ewes receiving MAO-std or MAO-5mis control morpholinos, respectively, but was substantially reduced ($p<0.025$) in MAO-env ewes (Table 5.1). All monovulatory ewes injected with MAO-env exhibited estrus at the expected time (17-18 days post-mating), which is indicative of early pregnancy loss due to inadequate IFNT production by the conceptus. At hysterectomy, a normal elongated, filamentous and implanting conceptus was observed in the ligated uterine horn of ewes injected with control morpholinos (data not shown).

In order to complement the *in vivo* data from Studies One and Two, we isolated mononuclear trophectoderm cells from day 15 conceptuses and cultured them *in vitro*. As assessed by RT-PCR analysis, the cultured mononuclear trophectoderm cells expressed enJSRVs *env* and *gag* mRNA as well as *IFNT* mRNA, while *CSH1* and *PAG* mRNAs were not detected (data not shown).

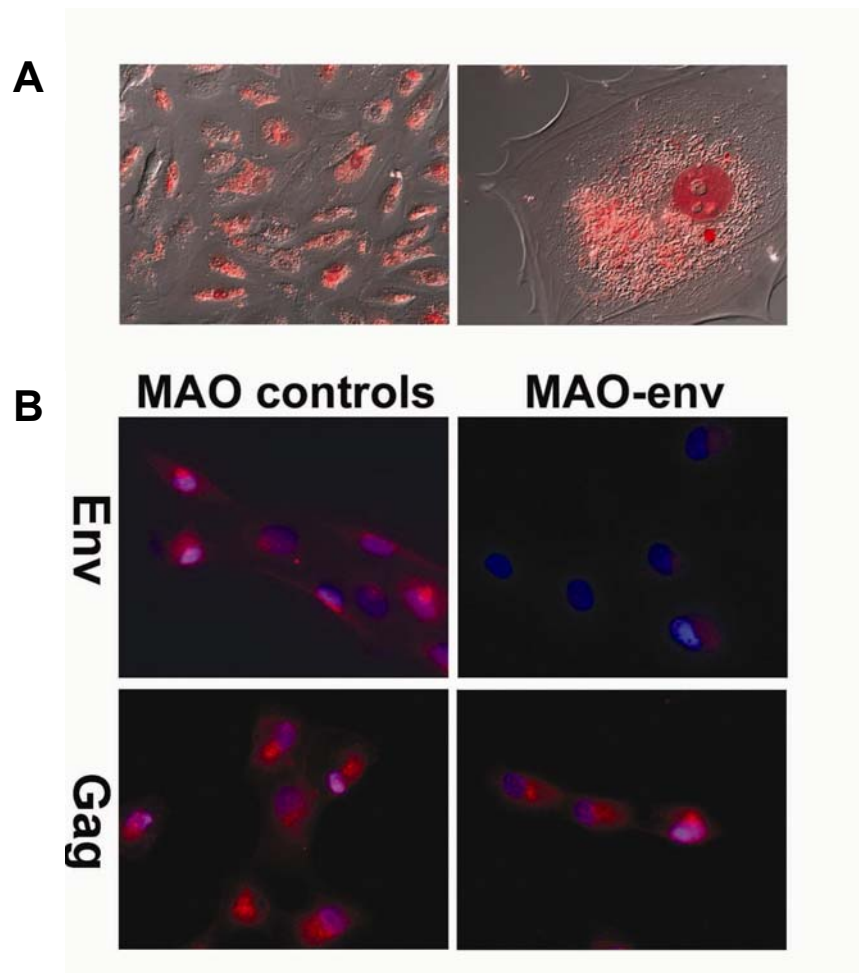


Figure 5.6. In vitro delivery and effectiveness of morpholinos. Mononuclear trophoblast cells were isolated from day 15 conceptuses. **(A)** Morpholino delivery. Rhodamine-labeled MAO-std was complexed with Endo-Porter aqueous delivery reagent and added to cells in culture. Fluorescence microscopy was used to visualize the labeled MAO in cells. Width of each field of view is equal to 870 μm on the left and 90 μm on the right. **(B)** Effect of morpholinos on enJSRVs Env and Gag protein in cultured trophoblast cells. Cells were grown on glass slides and mock-treated or treated with MAO-std, MAO-5mis or MAO-env for 48 h. Immunofluorescence analysis determined that enJSRVs Env but not Gag protein was inhibited in cells treated with MAO-env but not the other morpholinos. Results are representative of three experiments. Width of each field of view is equal to 140 μm .

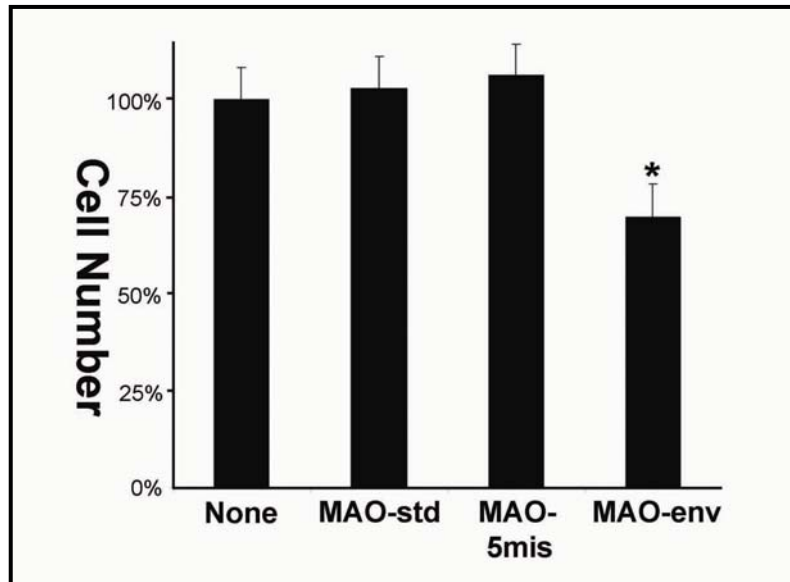


Figure 5.7. Effects of morpholinos on *in vitro* ovine trophectoderm proliferation Cells were grown in culture dishes until 30% confluency and mock-treated (no morpholino) or treated with MAO-std, MAO-5mis or MAO-env for 48 h. Cell number was reduced ($p<0.05$) by 33% in cultures treated with the MAO-env relative to control morpholinos. Results are from three independent experiments, and data is expressed as the percentage of cell number in mock-treated cultures.

Morpholinos were effectively delivered to over 95% of the trophectoderm cells *in vitro* (Figure 5.6A). Immunofluorescence analyses found that treatment of trophectoderm cells *in vitro* with MAO-env inhibited expression of the enJSRVs Env, whereas the MAO-std and MAO-5mis controls had no effect on enJSRVs Env abundance (Figure 5.6B). As expected, none of the morpholinos affected enJSRVs Gag expression (Figure 5.6B). However, the MAO-env decreased trophectoderm cell proliferation (Figure 5.7). Trophectoderm cell number was reduced ($p<0.05$) by 33% in cultures treated with the MAO-env, whereas MAO-std and MAO-5mis had no effect.

Discussion

These results are the first to demonstrate an essential role for ERVs in placental morphogenesis by *in vivo* experimentation. Using a morpholino loss-of-function approach, the enJSRVs Env knockdown caused a reduction in trophectoderm outgrowth during blastocyst elongation and formation of the conceptus. IFNT is a developmentally regulated gene that is expressed only in the mononuclear trophectoderm cells of the sheep conceptus [143] from day 10 to day 20 with a peak on day 16 of pregnancy [144]. Thus, a reduced amount of IFNT was found in the uterine flushes of MAO-env treated ewes containing a growth-retarded conceptus with lower numbers of mononuclear trophectoderm cells.

In sheep, pregnancy recognition and establishment involves elongation of the spherical blastocyst to a filamentous conceptus between Days 12 and 16 and production of IFNT by the conceptus [145, 146]. IFNT is antiluteolytic and acts on the endometrium to inhibit development of the luteolytic mechanism, thereby maintaining corpus luteum function and ensuring continued production of progesterone [82]. Progesterone acts on the uterus to stimulate and maintain endometrial functions necessary for conceptus growth, implantation, placentation and development to term [147]. Thus, the pregnancy loss observed before day 20 in MAO-env treated ewes was likely caused by an inability of the growth-retarded conceptus to produce sufficient IFNT to abrogate development of the endometrial luteolytic mechanism, resulting in luteolysis and a return to estrus .

The *in vivo* loss-of-function studies presented here strongly support the hypotheses that enJSRVs play a fundamental role in mononuclear trophoblast cell outgrowth and possibly differentiation into trophoblast giant BNC during the peri-implantation period of pregnancy . In fact, little is known of the cellular and molecular mechanisms regulating trophoblast proliferation and differentiation during early pregnancy in ruminants . Moreover, the cellular and molecular mechanism(s) whereby enJSRVs Env has biological effects within cells is unknown. It is of interest that the JSRV *env* (the exogenous counterpart of enJSRVs) encodes a functional structural protein that is a dominant oncoprotein, a unique feature among oncogenic retroviruses . Thus, it is possible that enJSRVs and JSRV Env proteins share common mechanisms by which they influence the cell cycle and cell proliferation, but only the Env of the exogenous JSRV is truly oncogenic.

It is also possible that enJSRVs Env are essential for trophoblast giant BNC and formation of multinucleated syncytia by eliciting cell-cell fusion similar to proposed actions of syncytins in humans and mice (15-20). However, results of Study One clearly indicate that enJSRVs Env influences mononuclear trophoblast cell growth during blastocyst elongation that precedes formation of multinucleated syncytia. Future studies will determine if HYAL2 is involved in trophoblast giant BNC differentiation, because HYAL2 is expressed only in BNC and syncytia of the ovine placenta [7] and can serve as a receptor for the enJSRVs Env as well as the exogenous JSRV Env [64, 67]. It is tempting to speculate that HYAL2 is involved in syncytia formation , because enJSRVs Env are expressed in the mononuclear trophoblast cells and uterine luminal

epithelium whereas HYAL2 is solely expressed in the BNC that apparently fuse with the luminal epithelium and each other to generate the multinucleated syncytia . Further support for this hypothesis is that human and mouse syncytins, products of an *env* genes, are fusogenic when expressed in vitro.

From an evolutionary point of view, we speculate that the protection of the host against related exogenous retroviruses was a driving force influencing the fixation of ERVs in the genome of various mammals. For example, enJSRVs interfere with exogenous JSRV at both early and late steps of the replication cycle . After fixation of ERVs in the germline of the host, their expression in the placenta may have favored conceptus development and increased reproductive efficiency. Some of the host mechanisms governing these reproductive processes may have been lost later during evolution. In conclusion, available evidence obtained in vivo in sheep and in vitro in primates and rodents strongly support the hypothesis that independently acquired ERVs were positively selected for a convergent physiological role in placental morphogenesis. The enormous structural variability of placentae among major taxa supports a model where retroviruses have conferred increased diversity and functionality during evolution.

CHAPTER VI

SUMMARY

The observation in many animal species, including humans, of numerous ERVs expressed in the placenta and uterus suggested that these elements played a role in mammalian placental morphogenesis[26, 29, 32, 104, 148, 149]. This hypothesis is further supported by the discovery that the envelope glycoproteins of some human ERVs, such as HERV-W, are expressed in the placental syncytiotrophoblast and induce syncytia in vitro[84]. However, studies suggesting a potential link between ERVs and mammalian reproduction have been limited to in vitro systems[7]. The present studies characterize the expression of ovine enJSRVs and their receptor, *HYAL2*, mRNAs and protein throughout gestation. They further describe in vivo experiments that provide evidence for enJSRVs regulating conceptus growth and placental morphogenesis.

Results of Study One demonstrated that enJSRVs *env* was expressed beginning in the Day 12 conceptus, whereas *HYAL2* was expressed from Day 16. *HYAL2* mRNA was detected throughout gestation in the placentome, but not in the endometrium, whereas enJSRVs *env* expression was detected throughout gestation in endometrium and placentomes. The enJSRVs *env* mRNA was specifically expressed in the endometrial LE and GE as well as the trophoblast giant binucleate cells and multinucleated syncytia of the placenta. *HYAL2* mRNA was only detected in the BNC and multinucleated syncytial plaques of the placentome. Partial sequencing of the transcriptionally active enJSRVs from sheep endometrium, placentomes and placenta revealed many enJSRV loci. Cloning of the expressed enJSRVs *env* mRNA from ovine uteroplacental tissues

revealed sequences similar to the previously identified enJS5F16 and enJS56A1 gene with an intact open reading frame, although the polypeptides for which they encode were not studied. Collectively, results of the present study provide further support for the hypothesis that the enJSRVs Env are beneficial to the host and are involved in protection of the uterus from viral infection and in regulating placental morphogenesis and function.

Investigation of the effects of progesterone and interferon tau on enJSRV expression *in vivo* indicates that enJSRVs expression is directly regulated by progesterone and progesterone receptor in ovine endometrial epithelia. *In situ* hybridization analyses of uteri revealed that enJSRV RNA expression levels were lower in endometrial LE and GE of ewes that received the progesterone receptor antagonist (ZK) as compared to ewes that received progesterone only. No effects of IFN τ on enJSRVs expression were detected in sheep receiving either P alone or P+ZK. These *in vivo* results confirm those from *in vitro* [76] experiments indicating that progesterone, acting through PR, increases expression of enJSRVs in the endometrial LE and GE in a temporal manner coincident with the beginning of conceptus elongation and implantation.

We report here *in vivo* and *in vitro* experiments finding that the envelope of a particular class of ERVs of sheep (enJSRVs) regulates trophoctoderm growth and differentiation in the peri-implantation conceptus (embryo/fetus and associated extraembryonic membranes). The enJSRVs envelope gene is expressed in the trophoctoderm of the elongating ovine conceptus after Day 12 of pregnancy. Loss-of-

function experiments were conducted *in utero* by injecting morpholino antisense oligonucleotides on day 8 of pregnancy that blocked enJSRVs envelope protein production in the conceptus trophoctoderm. This approach retarded trophoctoderm outgrowth during conceptus elongation and inhibited trophoblast giant binucleate cell differentiation on day 16. Pregnancy loss was observed by day 20 in sheep receiving morpholino antisense oligonucleotides. Indeed, inhibition of enJSRVs Env *in vitro* reduced proliferation of isolated mononuclear trophoctoderm cells from day 15 conceptuses. Consequently, these results demonstrate that the enJSRVs envelope regulates trophoctoderm growth and differentiation in the peri-implantation ovine conceptus. These novel *in vivo* studies provide strong support for the biological significance of ERVs in placental morphogenesis and mammalian reproduction.

In ewes, the morphological aspects of binucleate cell differentiation are well characterized; however, the cellular and molecular mechanisms regulating their differentiation and development are not yet understood. Indeed, enJSRV env and HYAL2 mRNAs are co-expressed in the binucleate cell and multinucleated syncytiotrophoblasts throughout gestation. Figure 6.1 illustrates our working hypothesis regarding the mechanism(s) whereby enJSRV Env and HYAL2 regulate binucleate cell fusion and formation of a multinucleated syncytium.

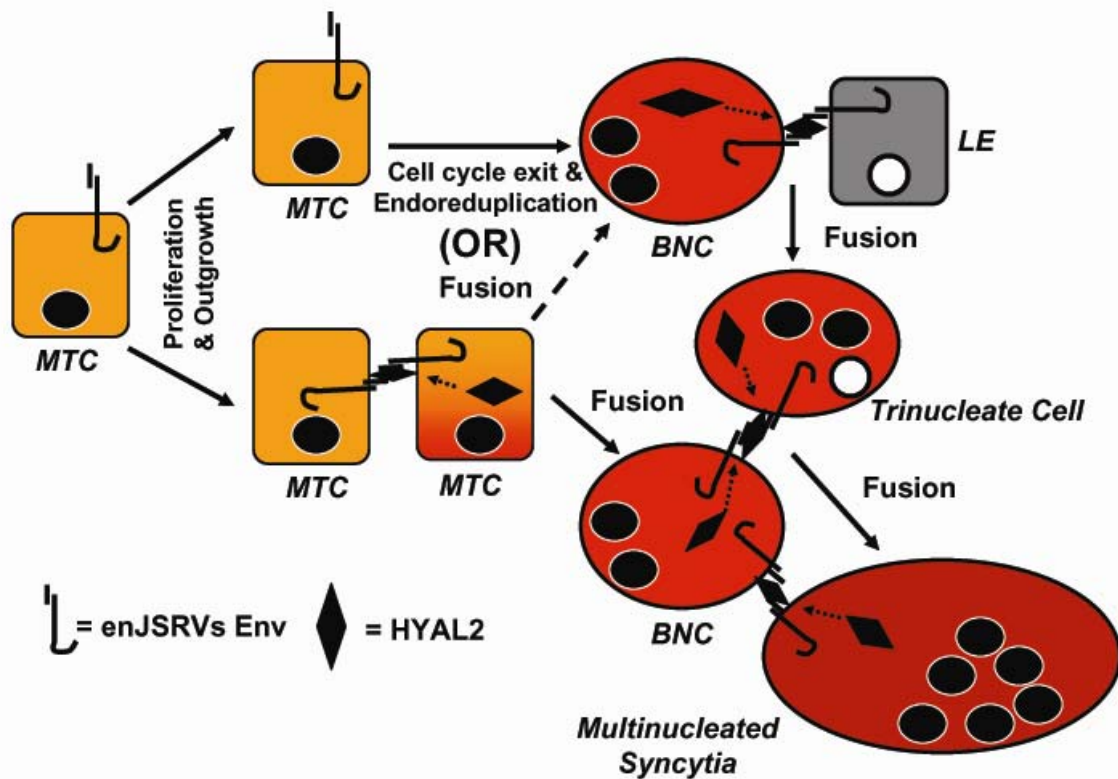


Figure 6.1. Model of multinucleated syncytia formation. Available results support enJSRVs Env hypothesized role in regulating: (i) mononuclear trophoblast cell proliferation and growth; (ii) trophoblast giant BNC differentiation; and (iii) formation of multinucleated syncytia.

During the peri-implantation period of placentation the conceptus elongates between days 12 and 16 and involves increased proliferation, growth, and remodeling of the mononuclear trophoblast cells (MTC). Some of the MTC begin to differentiate into trophoblast giant BNC between days 14 and 16. Histological evidence suggests that some BNC migrate and fuse with the uterine luminal epithelium (LE) [57, 62, 71, 123, 150]. Multinucleated syncytial plaques are formed by BNC fusion and may contain up

to 25 nuclei. Our published studies indicates that the onset of trophoblast outgrowth during conceptus elongation coincides with the enJSRV Env expression in the MTC and abundant enJSRVs Env in the trophoblast giant BNC and multinucleated syncytia throughout gestation [7]. Our in vivo studies found that inhibition of enJSRVs Env protein in the conceptus retarded trophoblast outgrowth and inhibited BNC differentiation on day 16. Consequently, pregnancy establishment was compromised by day 20 from reduced IFN τ (the hormone responsible for pregnancy recognition) due to lower numbers of MTC as well as lack of implantation due to absence of BNC.

Thus, enJSRV Env is hypothesized to regulate: (i) mononuclear trophoblast cell proliferation and growth; (ii) trophoblast giant BNC differentiation; and (iii) formation of multinucleated syncytia. Available results support the working hypotheses that enJSRVs Env: (i) modulate one of many critical signaling pathways necessary for MTC proliferation and growth, such as MAPK and PI3K/Akt; and (ii) may furnish the main driving force for the differentiation of MTC into BNC cells and subsequent formation of multinucleated syncytia. Given that HYAL2 is first expressed in the conceptus between days 14 and 16, it is tempting to speculate that HYAL2 is involved in trophoblast BNC differentiation due to the ability to bind enJSRVs Env. Collectively, it appears that ERVs play critical roles in conceptus growth, cell fusion, and placental differentiation in mammals and the generation of fundamental knowledge concerning the cellular and molecular mechanisms of trophoblast growth and differentiation as well as unraveling basic mechanisms of cell-cell fusion are important areas of emphasis for future research.

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